Characterization and encapsulation of probiotic bacteria using a pea-protein alginate matrix

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ABSTRACT

Research was undertaken to examine different *in vitro* characteristics of probiotic bacteria, including *Lactobacillus acidophilus* ATCC® 11975™, *Bifidobacterium infantis* ATCC 15697D, *Bifidobacterium catenulatum* ATCC® 27675 and *Bifidobacterium adolescentis* ATCC® 15703™ in order to identify suitable strain(s) for encapsulation. Under simulated gastric conditions (pH 2.0), *L. acidophilus* was the most acid-tolerant strain (*D*-value 10.2 ± 0.8 min), and was able to survive for 30 min; whereas, the other tested probiotics underwent a rapid (within the first 5 min at pH 2.0) 4-5 log cfu/mL loss in viability. All probiotics tested were able to survive 5 h exposure to 0.3% Oxgall bile at pH 5.8. The relative ranking of probiotic adherence to Caco-2 cells was determined to be: *L. acidophilus* >*B. catenulatum* >*B. adolescentis* >*B. infantis,* which correlated with 4.5×10^4, 3.1×10^3, 2.6×10^1, and 1.5×10^1 cfu/mL associated with Caco-2 cell monolayers, respectively. The most hydrophobic probiotics included *L. acidophilus* (46.5 ± 6.1%) and *B. catenulatum* (65.5 ± 5.2%); their hydrophobicity were positively correlated with auto-aggregation ability. Addition of divalent cations, EDTA, and bile salts were found to affect hydrophobicity as well; for example, 0.5 mM $MgCl_2$ resulted in a 20% increase in cell surface hydrophobicity of *L. acidophilus* from baseline levels; whereas, the addition of 0.1 and 0.5% bile salts decreased *L. acidophilus* hydrophobicity from control levels by 60 and 90%, respectively. Cell free culture supernatant of *L. acidophilus* effectively inhibited the growth of *Escherichia coli* O157:H7, and *Clostridium sordelli*. Bactericidal activity of *L. acidophilus* cell-free supernatant (the lethal factor was determined to be both heat and trypsin-resistant) against *Escherichia coli* O157:H7 and *Clostridium sordelli* ATCC 9714 over 24 h resulted in reductions of 5.5 and 3.5 log cfu/mL, respectively. Further examination of probiotics revealed varying degrees of resistance to the antimicrobial agents ciprofloxacin (4

μg/mL), naladixic acid (32 μg/mL), kanamycin (64 μg/mL) and sulfisoxazone (256 μg/mL). Determination of carbon source utilization patterns indicated that *B. catenulatum* utilized a number of carbohydrates including β-methyl-D-glucoside, D-xylose, D-cellobiose, and α-D-lactose; whereas, *L. acidophilus*, *B. infantis*, and *B. adolescentis* utilized D-xylose. *L. acidophilus* was ultimately selected for encapsulation in a 3 mm diameter pea protein-alginate matrix followed by *in vitro* challenge to simulated gastric conditions (pH 2.0). Encapsulation of *L. acidophilus* demonstrated a significant ($P < 0.05$) protective effect during the 2 h exposure to simulated acidic stomach conditions; within capsules, there was approximately 1 log cfu/mL loss in cell viability, whereas unprotected cells experienced >6 log/mL loss in cell viability over the same period.

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DEDICATION

This dissertation is dedicated to my beloved parents Seetarama Murty and Annapurna, husband K. C. Mouli and daughter Srinidhi for their understanding, love and moral support throughout the course of the program.

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1 General introduction

Probiotics are microorganisms introduced orally in the gastrointestinal tract (GIT) that are able to contribute positively to the activity of intestinal microflora and therefore, to the health of its host. Most probiotic bacteria belong to the group of lactic acid bacteria (LAB) and among them lactobacilli and bifidobacteria reportedly play a significant role in maintaining the intestinal ecosystem and in stimulating the immune system of the host (Saarela et al., 2002). Many *in vitro* properties, such as adhesion, resistance to pH, etc., are usually investigated to determine if a specific selected strain would be suitable as a probiotic (Collins et al., 1998).

Co-aggregation is a process by which genetically-distinct bacteria adhere to one another via specific molecules. Cumulative evidence suggests that such adhesion influences the development of complex multispecies biofilms (Ricard et al., 2003). Bacterial aggregation between microorganisms of the same strain (auto-aggregation) or between genetically divergent strains (co-aggregation) is of considerable importance in several ecological niches, especially inH the human gut, where probiotics are to be active (Collado et al., 2007a). A relationship between auto-aggregation and adhesion ability has been reported for some bifidobacterial spp. (Del Re et al., 2000; Collado et al., 2007b). A correlation between adhesion ability and hydrophobicity, as measured by microbial adhesion to hydrocarbons, has also been observed in some lactobacilli (Del Re et al., 2000). Furthermore, it has been suggested (Collado et al., 2007b) that inhibitor- or bacteriocin-producing LAB, which co-aggregates with pathogens, may constitute an important host defence mechanism against infection. Co-aggregation with potential gut pathogens could therefore contribute to the probiotic properties ascribed to specific LAB.

The determination of antimicrobial susceptibility of a bacterial strain is an important prerequisite for its approval as a probiotic (Moubareck et al., 2005). The *resistance gene*

reservoir hypothesis (Ammor et al., 2007) suggests that beneficial and commensal bacterial populations play a role in the transfer of antibiotic resistance to pathogenic and opportunistic bacteria. At present, there is great concern that commensal bacterial populations from food and the GIT of humans and animals, such as LAB and bifidobacteria, could act as a reservoir for antibiotic resistance genes. Resistance factors could ultimately be transferred to human pathogenic and opportunistic bacteria, thereby hampering the treatment of infections and general spread of antimicrobial resistance. LAB spp. have traditionally been used as starter cultures in the production of fermented feed and foodstuffs. Further, LAB and bifidobacteria are normal inhabitants of the GIT where they are known to exert health-promoting effects, and these selected strains are currently been used as probiotics. Antibiotic resistance genes carried by LAB and bifidobacteria could be transferred to human pathogenic bacteria either during food manufacture or during passage through the GIT (Ammor et al., 2007).

The overall goal of this research is to screen four probiotic bacteria, including *Lactobacillus acidophilus* ATCC 11975, *Bifidobacterium infantis* ATCC 15697, *B. catenulatum* ATCC 27675 and *B. adolescentis* ATCC 15703, for characteristics that make them effective candidates for encapsulation.

1.1 Hypotheses

The following hypothesis will be tested during this research:

a) Biochemical characterization of probiotic bacteria will identify candidates suitable for evaluation of probiotic encapsulation technology.

b) Probiotic encapsulation using pea protein-alginate beads will enhance probiotic survival in simulated gastric juice.

1.2 Technical objectives

The following objective will be investigated:

a) To examine a panel of probiotic bacteria for their ability to survive and or grow in low pH environments, as well as in the presence of bile salts;
b) To quantify the adherence of probiotic bacteria to intestinal epithelial surfaces, as well as their ability to co-aggregate with other enteric flora;
c) To determine the antimicrobial potential of probiotic bacteria against a panel of selected enteric pathogens;
d) To characterize probiotic bacteria using BIOLOG sole carbon source utilization, and antimicrobial profiling, to assist in strain-specific discrimination of the probiotic organism from other bacteria that colonize the gut environment; and
e) To utilize protein-alginate capsules to determine the effect of encapsulation of probiotic bacteria on survival in a model acidic (pH 2.0) gastric system.

2 Literature review

Foods are no longer considered by consumers only in terms of taste and immediate nutritional needs, but also in terms of their ability to provide specific health benefits beyond their basic nutritional value. Currently, the largest segment of the functional food market is provided by the foods targeted towards improving the balance and activity of the intestinal microflora (Saarela et al., 2002). Consumption of foods containing live bacteria is the oldest and still most widely used way to increase the numbers of advantageous bacteria in the intestinal tract. Such bacteria are called 'Probiotics' and have been predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, both of which have been extensively studied and established as valuable native inhabitants of the GIT (Fuller, 1989; Salminen et al., 1998, Capela et al.,

2006). Various microorganisms, particularly species of *Lactobacillus* and *Streptococcus*, have traditionally been used in fermented dairy products to promote human health as well as food functionality and flavor.

2.1 Historical perspective of probiotics

Escherich described the microbiota of the infant GIT and suggested benefits of their colonization in digestion. Around the same time, Doderlein postulated the beneficial association of vaginal bacteria in inhibiting the growth of pathogenic bacteria by producing lactic acid (Goktepe et al., 2006). Studies by Moro in 1900 and by Beijerinck in 1901 reported the beneficial association of LAB with human host (Goktepe et al., 2006). The longevity of Caucasians was related to the high intake of fermented milk products (Metschnikoff, 1907), as elucidated in his bestselling book *The Prolongation of Life*. LAB belong to a group of Gram-positive, non-sporulating, non-respiring cocci or rods, which produce lactic acid as a major metabolic end product during the fermentation of carbohydrates (Salminen et al., 1998). Although phylogenetically different, bifidobacteria are another group of lactic acid producing bacteria which are commonly accepted as LAB. Bifidobacteria were found to be typically associated with the feces of breast-fed infants and a lower incidence of intestinal upset was observed for breast-fed infants, when compared with formula-fed infants (Goktepe et al., 2006).

2.1.1 Definition of probiotics

The word probiotic is derived from the Greek meaning “for life”. Probiotics were first defined by Kollath in 1953 to denote all organic and inorganic food complexes in contrast to harmful antibiotics. Lilly and Stillwell (1965) defined probiotics as “microorganisms promoting the growth of other microorganisms”. Although numerous definitions have been proposed since

then, most have failed to be completely satisfactory because they lack statements such as "stabilization of the gut flora" (Goktepe et al., 2006). Havenaar and Veld (1992) have defined probiotics as "mono- or mixed cultures of live microorganisms which, when applied to animal or human, beneficially affect the host by improving the properties of the indigenous microflora". When these probiotic bacteria are present in yogurt and other fermented foods, they may beneficially alter the normal gut flora (Metchnikoff, 1907). Probiotics have also been defined by the European Union (EU) Expert Group on Functional Foods in Europe (FUFOSE) to be "viable preparations in foods or dietary supplements to improve the health of humans and animals" (FUFOSE working group, 1999). More recently, probiotics have been referred to as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001).

2.2 The gastrointestinal ecosystem

The GIT of the human body is a complex ecosystem with a diverse and concentrated microbial population that mediates numerous interactions with the chemical environment, such as digestion, adhesion and colonization in the GIT. The mucosal surface area increases by: circular folding which contributes to about a 3-fold increase, through the production of villi, for a 7- to 10-fold increase, and by the formation of intestinal microvilli, which results in a 15- to 40-fold increase (Holzapfel et al., 1998). Varying numbers of bacteria are found throughout the GIT, ranging from 10^1-10^3 cfu/mL or g in the stomach contents; 10^7 cfu/mL in the jejunum, up to 10^9 cfu/g in the terminal ileum and approximately 5×10^{11} cfu/g in the distal colon contents (Goktepe et al., 2006).

The bacteria detected in feces reflect the bacteria present in the distal colon, thus studies of the human GIT microflora usually involve analysis of fecal samples (Moore et al., 1978).

Traditional culture methods have been used to analyze and characterise microbial communities in a natural ecosystem to obtain a complete diversity picture. In contrast to the microaerophillic lactobacilli, the study of anaerobic bifidobacteria and eubacteria was made possible by the development of anaerobic techniques in the early 1970s (Goktepe et al., 2006). However, cultivation techniques have major limitations, as many microbes in different ecosystems cannot be cultivated by standard culture methods (Ward et al., 1990). Classical culture-independent techniques include direct microscopic analysis and monitoring specific enzymes or metabolites, and have provided valuable insight into the real numbers of microflora in faecal samples. Microscopic analysis of faecal samples by Langendijk et al. (1995) revealed approximately 10^{11} to 10^{12} organisms per g of wet feces. It is noteworthy that these techniques are very limited in their ability to give any in-depth characterisation of specific organisms present or community diversity. Fluorescence microscopy, confocal laser scanning microscopy and flow cytometry have been used to detect viable populations through the use of fluorescent probes (Lipski et al., 2001). If epifluorescence microscopy and/or confocal laser scanning microscopy are applied, the method is usually referred to as fluorescence *in situ* hybridization (FISH). FISH has been used to study the composition of GIT microbial system (Tannock et al., 2000). Various studies reported that the microscopic technique itself is not perfect and may significantly under-report the true numbers (Ward et al., 1990).

Various short chain fatty acids (SCFA), such as acetate, propionate and butyrate, are end products of anaerobic bacterial fermentation. Thus, measurement of these acids in feces can be correlated with specific bacterial metabolism in the intestine (Rowland, 1989). For example, *Lactobacillus casei* GG fed to children with an intestinal infection significantly increased the total SCFA concentration (Siigur et al., 1996). Increases or decreases in specific enzymes for

example, β-glucuronidase and β-galactosidase, in feces can also point to the metabolic activities of certain groups of bacteria. Reduction in β-glucuronidase levels was reported in humans during ingestion of *L. casei* GG (Ling et al. 1994). Also, a significant correlation has been observed between the levels of faecal β-galactosidase and numbers of bifidobacteria (Favier et al., 1997). While many faecal enzymes, such as azoreductase and nitroreductase are mainly produced by the species *Bacteroides, Eubacterium* and *Clostridium,* more studies are needed to accurately correlate specific faecal enzymes with specific groups of bacteria (Rowland, 1989).

Cell viability can also be inferred from enzymatic activities such as esterase conversion of carboxyfluorescein diacetate (cFDA). The reduction of tetrazolium salts, or dyes such as propidium iodide, TOTO-1, SYTO 9, carboxyfluorescein and oxonol, have been used as viability indicators (Goktepe et al., 2006). Bunthof et al. (2001) have combined culture plating technique with dyes, and reported that cFDA labels the culturable subpopulation; whereas, TOTO-1 lables the non-culturable population. Determination of percentages of guanine+cytosine (G+C) content is one of the few methods depicting the total bacterial community of the GIT without any previous knowledge of component bacteria or their DNA sequences (Apajalahti et al., 1998). This approach has been applied by Apajalahti et al. (2003) to determine the total bifidobacteria community in human feces.

DNA-based methods for the detection of probiotic microorganisms are mainly based on restriction enzyme analysis or PCR (Polymerase Chain Reaction), or both. Various methods including amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis (PFGE), Random amplification of polymorphic DNA (RAPD) including multiplex PCR, arbitrary primed PCR (AP-PCR) and tiplicate arbitrary-primed PCR (TAP-PCR) has been used for identification and tracking of individual probiotic strains (Gardiner et al., 2002). AFLP is a

combination of PCR and restriction enzyme analysis, where genomic DNA is digested with two different types of restriction endonucleases. Strain specific identification using AFLP method has been used to differentiate *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* (Torriani et al., 2001). PFGE has been used for typing *Lactobacillus casei*, bifidobacteria and *Lactobacillus rhamnosus* (Goktepe et al., 2006).

RAPD is a PCR-based method in which a pattern of amplicons is produced through the simultaneous amplification of many chromosomal sequences mediated by annealing of short oligonucleotide primers. RAPD PCR analysis has been reported to be capable of differentiating between *L. acidophilus* group strains (Pleiss et al., 1995). This technique has also been found to be useful for monitoring introduced and indigenous lactobacilli in the intestinal tract (Gardiner et al., 2002). RAPD PCR analysis of yeast isolates from feta cheese provided reliable identification at species level and good discrimination at the strain level (Psomas et al., 2001). In AP-PCR, reactions are performed by using specific primer targeting a highly conserved region within the 16S rRNA gene. TAP-PCR is a variation of AP-PCR, where three different annealing temperatures are used in triplicate reactions, and has been used by Cusick and O'Sullivan (2000) to type isolates from major genera of LAB and bifidobacteria. The primers used in the amplification reaction in Rep-PCR and ERIC-PCR techniques targets the species specific Rep (Repetitive extragenic palindromic) elements and ERIC (enterobacterial repetitive intergenic consensus) sequences, which are conserved regions dispersed on the genomic DNA of microorganisms. The profiles obtained due to the amplification of inter-Rep and inter-ERIC distances are species and sometimes strain specific (Goktepe et al., 2006).

Other culture-independent techniques which can mediate the identification of individual bacterial species or strains include denaturing gradient gel electrophoresis (DGGE), which has

been used to detect lactobacillus and other species in the human GIT (Walter et al., 2001). Accurate typing of unknown isolates is now achieved through sequence analysis of 16S ribosomal RNA (rRNA) or the corresponding rDNA amplicons following PCR. Matsuki et al. (2002) has investigated the microbial population of six healthy human volunteers by applying 16S rRNA-gene-targeted group-specific-oligonucleotide primers for the *Bacteroides fragilis* group, *Bifidobacterium*, the *Clostridium coccoides* group, and *Prevotella*, and identified 74% of the predominant bacteria in the feces. Another method called amplified ribosomal DNA restriction analysis (ARDRA) has been developed on the basis of 16s rRNA gene amplification followed by restriction analysis, and was used by Ventura et al. (2000) to identify different species of *Lactobacillus* isolated from human feces and vagina.

Andersson et al. (2008) has developed a method based on 454-pyrosequencing for monitoring of microbial communities in throat, stomach and fecal samples. Pyrosequencing is a method of DNA sequencing based on the sequencing by synthesis, which involves taking a single strand of the DNA to be sequenced and then synthesizing its complementary strand enzymatically. A highly variable region of the 16S rRNA gene is amplified using primers that target adjacent conserved regions, followed by direct sequencing of individual PCR products. The cpn60 gene (encoding the universally conserved 60 kDa chaperonin), has been established as a useful target for molecular phylogenetics, characterization of complex microbial communities and to differentiate between closely related bacterial isolates by hybridization or sequence analysis (Dumonceaux et al. 2006). An approximately 555-bp segment of the gene corresponding to nucleotides 274–828 of the *Escherichia coli* cpn60 sequence (the cpn60 universal target, or cpn60 UT) can be amplified from virtually any genome using universal, degenerate PCR primers. The cpn60 based identification of fecal microflora of cats has revealed

diverse populations dominated by Actinobacteria (particularly bifidobacteria) and Firmicutes (particularly lactobacilli) (Desai et al. 2009).

2.2.1 Gastrointestinal strains of human origin

In spite of increased research on gut microbial ecology, only a small number of approximately 400 species of different genera have been cultivated and studied with regard to their physiology, metabolic interactions, and taxonomy (Goktepe et al., 2006). Table 2.2.1 presents the LAB found likely to be associated with the human host (Goktepe et al., 2006). The large intestine is densely populated by *Bacteriodes* and the Gram-positive, anaerobic genera *Eubacterium* and *Bifidobacterium.* Lactobacilli are the predominant species in the vagina and are also normally present in the oral cavity (10^3-10^4 cfu/g), the ileum (10^3-10^7 cfu/g), and colon (10^4-10^8 cfu/g), where they play an important role in maintenance of a stable gut mucosa (Lidbeck et al., 1993). Long and Swenson (1977) showed that bifidobacteria and lactobacilli are the dominant bacterial species found to be present in the feces of breast-fed infants. There is a lack of research evidence that has demonstrated a single dominant species in the human GIT. However, *L. acidophilus* (commonly referred as simply "*acidophilus*") has been recovered in relatively high numbers from the GIT (Molin et al., 1993). Strains of *acidophilus* have been isolated from the intestinal tract of humans as well as animals such as rodents and birds.

LABs are gram-positive, non-spore forming, catalase-negative organisms that are devoid of cytochromes and anaerobic but aerotolerant. They are fastidious, acid-tolerant, and strictly fermentative (either homo- or hetero); lactic acid is the major end product of sugar fermentation (Axelsson, 1998). However, some species can form catalase or cytochromes on media containing hematin or related compounds and some lactobacilli can also produce non-heme catalase, called pseudocatalase, which cause confusion for LAB identification (Holzapfel et al., 2001).

Table 2.2.1 LAB typically associated with the human host (Goktepe et al., 2006)

Lactobacilli	**Other LAB**
Intestinal Bacteria	
Lactobacillus acidophilus group	*Bifidobacterium adolescentis*
L. acidophilus senso strictu	*B. angulatum*
L. animalis	*B. bifidum*
L. brevis	*B. breve*
L. buchneri	*B. cantenulatum*
L.crispatus	*B. dentium*
L. curvatus	*B. infantis*
L. delrueckii	*B. longum*
L. fermentum	*B. pseudocantenulatum*
L. gasseri	*Enterococcus fecalis*
L. johnsonii	*E. faecium*
L. paracasei	*Leuc. Mesenteroides*
L. plantarum	*Pedicoccus pentosaceus*
L. reuteri	*Weissella confusa*
L. rhamnosus	
L. ruminis	
L. sakei	
Vaginal Bacteria	
Lactobacillus acidophilus	*Bifidobacterium bifidum*
L. fermentum	*B. longum*
L. casei	*B. infantis*
L. rhamnosus	*B. breve*
L. cellobiosus	*B. catenulatum*
L. plantarum	*B. dentium*
L. brevis	
L. delbrueckii	
L. salivarious	
L. jensenii	
L. vaginalis	
L. gasseri	
L. crispatus	

2.3 Probiotic bacteria

Strains of LAB, such as *Lactobacillus, Bifidobacterium, Eubacterium* and *Streptococcus*, have traditionally been used in the manufacture of fermented dairy products and are generally regarded as safe (GRAS) (O'Sullivan et al., 1992). In addition, these bacteria are desirable members of the intestinal microflora (Berg, 1998). Table 2.3.1 shows a list of microorganisms

including both LAB and non-lactics which are generally considered as probiotics. Lack of pathogenicity, tolerance to gastrointestinal conditions (acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens (Collins et al., 1998; Ouwehand et al., 2002) are some of the general criteria that have been used for the selection of probiotics. *L. casei* strain "Shirota" has been reported to have the longest history of safe use as a probiotic in food with proven health benefits (Goktepe et al., 2006). *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were used to make mildly acidified yogurts called "bio-yogurts" in Germany during late 1960s (Goktepe et al., 2006). Viable probiotic strains with beneficial functional properties are supplied in the market as fermented food products, mainly "yogurt"-type, or in lyophilized form, both as food supplements and as pharmaceutical preparations. For many years, pharmaceutical preparations containing live microorganisms in capsules, also known as "biotherapeutics", were used for the restoration of the GIT population, e.g., after or during antibiotic treatment (Goktepe et al., 2006).

The prevalence of lactobacillus and bifidobacterial spp. in the intestinal tract of humans is not known accurately. *Lactobacillus crispatus, L. gasseri, L. salivarius,* and *L. reuteri* have been reported as the major species of the *Lactobacillus* microflora (Mitsuoka et al., 1990). Whereas, *Lactobacillus johnsonii*, *Lactobacillus ruminis*, *Lactobacillus casei*, and *Lactobacillus brevis* have been detected occasionally. *Bifidobacterium longum* has been found predomonently in adult human GIT, while *Bifidobacterium bifidum* was detected occasionally. In contrast, *Bifidobacterium infantis* and *Bifidobacterium breve* were detected predominantly in infant feces, while *B. longum* and *B. bifidum* detected occasionally (Biavati et al., 1984).

Table 2.3.1 Microorganisms considered as probiotics (Adapted from Holzapfel et al., 2001)

Lactobacillus spp.	***Bifidobacterium spp.***	**Other LAB**	**"Non-lactics"** [a]
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis* [a]	*Bacillus cereus* ("toyoi") [a,c]
L. amylovorus	*B. animalis*	*Ent. faecium*	*Escherichia coli*[a]
L. casei	*B. bifidum*	*Sporolactobacillus inulinus*[c]	*Propionibacterium freudenreichii* [a,c]
L. crispatus	*B. breve*		*Saccharomyces cerevisiae* ("boulardii") [a]
L. delbrueckii subsp. bulgaricus [a]	*B. infantis*		
L. gallinarum [c]	*B. lactis* [b]		
L. gasseri	*B. longum*		
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

[a] Mainly in pharmaceutical preparations; [b] Synonym of *B. animalis;* [c] Mainly for animals.

On the basis of their morphologic and phenotypic features, the LAB are subdivided (Table 2.3.2) into the genera *Betabacterium, Thermobacterium, Streptobacterium, Streptococcus, Betacoccus, Tetracoccus,* and *Microbacterium* (Holzapfel et al., 2001). *Enterococcus, Lactococcus,* and *Vagococcus* have been separated from the original genus, *Streptococcus* (Holzapfel et al., 2001). The genus *Streptococcus* represents mainly pathogenic bacteria, except *Streptococcus thermophilus*; whereas, some strains of *Enterococcus* spp. may be involved in opportunistic infections, and some are considered to play role in food fermentations, and are also found as commensals in the GIT. *Lactococcus* spp. are generally considered to be non-pathogenic and safe.

Probiotic *Bifidobacterium* spp. are generally strict anaerobes. Fermentation of the sugars and sugar alcohols like L-arabinose, D-xylose, D-mannose, salicin, D-mannitol, D-sorbitol, and D-melezitose serve as key characteristics to identify the most important species of bifidobacteria (Klein et al., 1998).

Table 2.3.2 Key to differentiating lactic acid bacteria and a comparison with current taxonomic classification (Adapted from Holzapfel et al., 2001)

Genus	Shape	Catalase	Nitrite reduction	Fermentation	Current genera
Betabacterium	Rod	−	−	Hetero-	*Lactobacillus* *Weissella*
Thermobacterium	Rod	−	−	Homo-	*Lactobacillus*
Streptobacterium	Rod	−	−	Homo- and Hetero	*Lactobacillus* *Carnobacterium*
Streptococcus	Coccus	−	−	Homo-	*Streptococcus* *Enterococcus* *Lactococcus* *Vagococcus*
Betacoccus	Coccus	−	−	Hetero-	*Leuconostoc* *Oenococcus* *Weissella* *Brochothrix*
Microbacterium	Rod	+	+	Homo-	*Pediococcus*
Tetracoccus	Coccus	+	+	Homo-	*Tetragenoccus*

Analysis of the cell wall peptidoglycan composition was also found suitable for the identification of some species like *Bifidobacterium longum, Bifidobacterium infantis,* and *Bifidobacterium suis* (Bonaparte, 1997). Practically all organisms used in probiotic foods or food supplements are representatives of the genera *Lactobacillus, Enterococcus,* or *Bifidobacterium*. The genus *Bifidobacterium* shares some phenotypic features with typical LAB and is considered to form part of the LAB. Phylogenetically-distinct, bifidobacteria exhibit a relatively high G + C content of 55–67 mol% in the DNA and form part of the *Actinomycetes* branch. The "true" LAB form part of *Clostridium* branch, which is characterized by a G + C content of <55 mol% in the DNA. Genes encoding rRNA, comprising conserved and variable domains, are typically chosen for phylogenetic work as they are present in all microorganisms. Analysis of the 16s rRNA gene is considered to be the most powerful and accurate technique for determining the degree of phylogenetic relation of microorganisms.

The phylogenetic relation of the different genera of "true" LAB is shown in Figure 2.3.1 and is based on the comparison of 16S rRNA sequences. *Carnobacterium*, *Enterococcus*, *Vagococcus*, *Aerococcus*, *Tetragenococcus*, and *Lactosphaera* are more closely related to each other than to any other LAB. Lactobacilli are phylogenetically diverse, whereas *Lactococcus* and *Streptococcus* are closely related. *Lactobacillus* and *Pediococcus* are mixed, phylogenetically, as shown by the 16S rRNA sequencing data with 5 species of a *Pediococcus* clustering with 32 homo- and hetero-fermentative *Lactobacillus* spp. in the so-called *Casei* and *Pediococcus* group (Collins et al., 1991). In 16S rRNA sequence data of pediococci and lactobacilli, the taxa generated do not correspond with the phylogenetic branching. Therefore, certain species of LAB may have to be reclassified.

Performing a preliminary *in vitro* assessment is a prerequisite to assess the properties of probiotic bacterial strains (FAO/WHO, 2002). Various papers (Dunne et al., 2001; Morelli, 2007) have suggested that a probiotic bacterial strain should be assessed according to the following (or a very similar) criteria: human origin, nonpathogenic behavior, resistance to technologic processes (i.e., viability and activity in delivery vehicles), resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, ability to persist within the gastrointestinal tract, production of antimicrobial substances, ability to modulate immune responses, and ability to influence metabolic activities (e.g., cholesterol assimilation, lactase activity, and vitamin production). It has also been suggested that the demonstration of probiotic activity of a certain strain involve well-designed, double-blind, placebo-controlled human studies (Dunne et al., 2001).

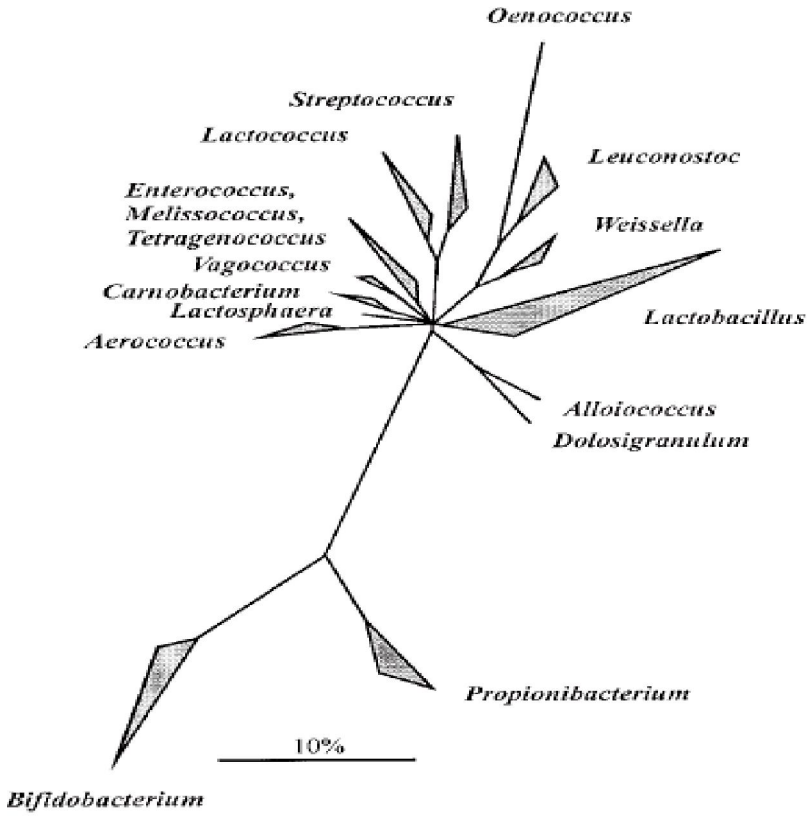

Figure 2.3.1 Consensus tree, based on comparative sequence analysis of 16S rRNA, showing major phylogenetic groups of lactic acid bacteria with low mol% guanine plus cytosine in the DNA and the non-related gram-positive genera *Bifidobacterium* and *Propionibacterium* (Adapted from Holzapfel et al., 2001)

Health benefits associated with the ingestion of probiotic bacteria includes: reduction in colon irritation, constipation, traveler's diarrhea, inhibition of the adhesion of pathogenic genera including *Escherichia, Clostridium, Salmonella* and *Campylobacter* to the intestinal lumen, synthesis of B vitamins, lowering of blood ammonia levels, cholesterol absorption and inhibition of tumor formation (Ziemer and Gibson, 1998). Some reported health benefits of probiotic bacteria are listed in Table 2.3.3.

Table 2.3.3 Some Probiotic bacterial and yeast strains and their reported health effect (Adapted from Dunne et al., 2001)

Strain	Reported effects
Lactobacillus acidophilus LC1	Immune enhancing, vaccine adjuvant, adherence to human intestinal cells, balancing of intestinal microflora
L. acidophilus NCFO1748	Lowering of fecal enzymes, prevention of radiotherapy-related diarrhea, treatment of constipation
L. rhamnosus GG	Prevention of antibiotic-associated, rotavirus, acute and *Clostridium difficile* diarrhea, antagonistic against carcinogenic bacteria
L. casei Shirota	Balancing of intestinal bacteria, lowering of fecal enzymes, inhibition of bladder cancer
L. gasseri	Fecal enzyme reduction, survival in the intestinal tract
Bifidobacterium bifidum	Treatment of rotavirus diarrhea, balancing of intestinal microflora
Saccharomyces boulardii	Prevention of traveler's diarrhea, prevention and treatment of *Cl. difficile* diarrhea

2.3.1 Acid resistance of probiotics

More than two liters of gastric juice with a pH as low as 1.5 is secreted from cells lining the stomach each day, providing a normally-effective, high-acid barrier against entrance of viable bacteria into the GIT. The effect of gastric pH on bacterial viability and in preventing bacterial colonization of the small intestine is well-studied (Simon and Gorbach, 1987; Heatley and Sobala, 1993). Consequently, any probiotic organism that is to survive transit through the stomach must have a high acid tolerance. In typical acid tolerance tests, the viability of candidate probiotic organisms is determined by exposing them to low pH in a buffer solution or medium for a period of time, during which the number of surviving bacteria remaining is determined.

Minekus et al. (1995) has developed a dynamic computer-controlled model, which reflects the *in vivo* conditions of the stomach and small intestine. This model permits an accurate simulation of the factors influencing the survival of probiotic microorganisms, such as pH, bile concentration and transit through the different parts of the GIT. Studies by Dunne et al. (2001) suggested that lactobacilli isolated from human ileal samples could successfully transit the human stomach conditions and function effectively. In these studies, bifidobacteria were found to be less resistant to stomach conditions like low pH etc. than lactobacilli. Studies by Conway et al. (1987) showed that yogurt-producing species of lactobacilli were more sensitive to gastric juice while enteric species were more resistant. The best-performing among the two *L. acidophilus* strains (strain ADH) used in the study were reclassified as *Lactobacillus gasseri*, which is a homofermentative lactobacilli (Morelli, 2000). *Lactobacillus rhamnosus* GG was unable to survive at pH 1.0, but remained viable at pH 3.0 and higher (Goldin et al., 1992). *In vitro* studies (Hood and Zottola, 1988; Charteris et al., 1998a) showed that enteric lactobacilli had a lower pH tolerance limit of 2.0 for several min. Eight meat starter cultures including *Lactobacillus* and *Pediococcus* strains were exposed to low pH (pH 1.0 to 5.0) conditions of stomach for 1 h. The number of surviving bacteria was decreased from the inoculated level of 7.4-7.6 log cfu/mL to < 4 log cfu/mL at pH 1.0 and pH 2.0, whereas pH 4.0 and 5.0 did not affect the viability (Erkkila and Petaja, 2000). Only 51 out of 312 pre-selected LAB strains, including *Lactobacillus, Pediococcus, Enterococcus,* isolated from Iberian dry fermented sausages, human and pig feces were able to survive after 1.5 h of exposure at pH 2.5, where the number of final surviving bacteria ranges between 5.4 to 8.9 log cfu/g (Ruiz-Moyano et al., 2008). The bile resistant isolates of *Bifidobacterium* strains displayed considerably higher survival at 90 min of exposure at pH 2.0, with a concentration of final surviving bacteria ~ 6.5 log cfu/mL, than their

corresponding strains of origin (Noriega et al., 2004). Survival of *Bifidobacterium animalis* strains BLC-1, Bb-12, and Bo, *Lactobacillus acidophilus* strains LAC-1 and Ki, *Lactobacillus paracasei* subsp. paracasei strain LCS-1 and *Lactobacillus brevis* strain LMG 6906 inoculated into whey cheese was assessed by Madureira et al. (2005). Except *L. paracasei* subsp. *paracasei* LCS-1 and *B. animalis* Bb-12, all bacteria were resistant to the action of artificial gastric juice (pH 2.5–3.0) and maintained their initial viable cell numbers (~ 8 log cfu/mL) after both 60 and 120 min of exposure.

In order to evaluate the survival of lactobacilli in the low pH conditions of the human stomach, five *Lactobacillus* strains were compared in simulated gastric juice (SGJ, pH 2.0) for 90 min (Corcoran et al., 2005). *Lactobacillus rhamnosus* GG had the highest survival rate and maintained their initial viable cell numbers (~ 9 log cfu/mL), while the poorest survivor was *L. paracasei* NFBC 338, whose concentration declined to undetectable levels after only 30 min of exposure. These studies also showed that glucose (19.4 mM) was responsible for the enhanced survival of *L. rhamnosus* GG in simulated gastric juice. The level of surviving bacteria was reduced by approximately 5.6 log cfu/mL upon removal of glucose (Corcoran et al., 2005). Furthermore, five *Lactobacillus* strains were examined for their acid tolerance (pH 2.5). *Lactobacillus acidophilus* NCFM was found to be the least acid tolerant, with a final surviving population of 6.0×10^4 cfu/mL, whereas the population of *L. acidophilus* 30SC and ATCC 43121 remained relatively constant (~ 6 log cfu/mL) (Oh et al., 2000).

Probiotics have been incorporated into a range of dairy products, including yoghurts, soft-, semi-hard and hard cheeses, ice cream, milk powders and frozen dairy desserts. However, there are still several problems with respect to the low viability of probiotic bacteria in GIT and food environments. Probiotics of intestinal origin are difficult to propagate and high survival is

important for both economic reasons and health effects. Consequently, there is a demand for new technologies such as encapsulation to enhance probiotic viability.

2.3.2 Bile resistance of probiotics

Bile is an aqueous solution made up of bile acids, cholesterol, phospholipids, and the pigment biliverdin, which gives the bile its yellow-green color. About 500-700 mL/day of bile acids are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum, after food intake by an individual (Hofmann and Roda, 1984). Bile plays an essential role in lipid digestion; it emulsifys and solubilizes lipids and functions as biological detergent. Prior to secretion into the duodenum, bile acids are conjugated either with glycine (glycoconjugated) or taurine (tauroconjugated) (Begley et al., 2006). In the colon conjugated bile undergoes various chemical changes including deconjugation, dehydroxylation, dehydrogenation, and deglucuronidation, almost solely by microbial activity (Begley et al., 2006). The antimicrobial nature of bile is mainly because of its detergent property, which dissolves bacterial membranes. Bile salt hydrolases (BSHs) are generally intracellular, oxygen-insensitive enzymes that catalyze the hydrolysis of bile salts. Hydrolysis of bile salts is mediated by various genera of the intestinal microflora, including *Clostridium* (Gopal et al., 1996), *Bacteroides* (Kawamoto et al., 1989), *Lactobacillus* (Lundeen and Savage, 1990; Christiaens et al., 1992), *Bifidobacterium* (Grill et al., 2000a) and *Enterococcus* (Franz et al., 2001). A number of BSHs have been identified and characterized in probiotic bacteria, and the ability of probiotic strains has often been included among the criteria for probiotic strain selection (Begley et al., 2006). Bile tolerance of probiotic bacteria can be investigated by incubating them for 24 hrs in a milk-yeast medium containing different concentrations of bile extracts and monitoring cell viability and pH before and after incubation (Goktepe et al., 2006). This assay was used by

several authors to assess the bile resistance of potential or already commercialized probiotic lactobacilli. All these studies reported a growth delay of lactobacilli in the presence of oxgall that was strain- and not species-dependent. It has been hypothesized that deconjugation of bile salts is a detoxification mechanism and BSH enzymes play a role in bile tolerance of probiotic organisms in the GIT (Savage, 1992). Both conjugated and deconjugated bile acids have been determined to inhibit the growth of *Klebsiella* spp., *Enterococcus* spp. and *Escherichia coli* strains *in vitro*. However, deconjugated forms of bile acids were found to be more inhibitory against Gram-positive than Gram-negative bacteria (Stewart et al., 1986). Studies by Smet et al. (1995) suggested that deconjugation of bile acids decreases their solubility and thus diminishes the detergent's activity and makes it less toxic to bacteria in the intestine. It was assumed that the conjugated form of the bile salts exhibits toxicity by causing intracellular acidification through the same mechanism as organic acid. In contrast, Tannock et al. (1989) stated that deconjugated bile salts are more inhibitory than conjugated bile salts to anaerobes including lactobacilli. Similarly, deconjugated bile was reported to be involved in growth inhibition of *Bifidobacterium* spp. including *B. breve, B. longum,* and *B. coryneforme,* where the viable counts were reduced by approximately 6, 7 and 2 log cfu/mL respectively, after 2 h incubation in the presence of 1 mM deconjugated bile (Grill et al., 2000b). Another hypothesis states that certain *Clostridium* spp. utilize the amino acid taurine as an electron acceptor and have demonstrated improved growth rates in the presence of taurine and taurine-conjugated bile salts (Moser and Savage, 2001). However, taurine or taurine conjugates did not affect the growth of *Lactobacillus* spp. tested (Tannock et al. 1989). Cholic acid was found to accumulate in lactobacillus cells by means of a transmembrane proton gradient (Kurdi et al., 2000). Whereas studies by Boever et al. (2000) reported that cholic acid was highly deleterious for the viability of lactobacilli. It has also been

suggested that the BSH enzymes are detergent shock proteins (Adamowicz et al., 1991) that protects the lactobacilli from its toxic effects and may have a competitive advantage over the non-BSH producing bacteria. However, studies of Moser and Savage (2001) reported that deconjugation and resistance are unrelated activities. Lastly, studies done by Gopal et al. (1996) showed no relationship between the ability of 6 strains of *L. acidophilus* and 8 strains of *Bifidobacterium* spp. to grow in bile (0.3% oxgall) and their ability to hydrolyze bile salts (glycocholic acid or taurocholic acid).

A link between bile salt hydrolysis and bile tolerance has been provided by the studies conducted on wild-type and *bsh* mutant pairs of *Lactobacillus plantarum, Lactobacillus amylovorus* and *Listeria monocytogenes.* Results showed that mutant cells were significantly more sensitive to bile and bile salts and displayed decreased growth rates in the presence of bile salts (Begly et al., 2006).

2.3.3 Probiotic adhesion to human intestinal cells

It is generally agreed that LAB must adhere to intestinal mucus or epithelial cells in order to persist in the gut. The ability of LAB to adhere to mucosal surfaces prevents their rapid removal by gut contraction and subsequent peristaltic flow of digesta, and could also confer a competitive advantage. A large body of research has been conducted to screen probiotic bacteria for their ability to attach to intestinal cells (Goktepe et al., 2006). *In vitro* experimentation shows that some strains of *Lactobacillus* adhere to intestinal tissue cultured cells in a species-dependent way (Fuller, 1975). However, other studies concluded that the capacity to adhere to the surface is undoubtedly insufficient by itself to ensure that the microorganisms can colonize the epithelial habitat (Savage, 1984). Cultured human intestinal cell line models, which express various specific characteristics of cell phenotypes of intestinal epithelium, have been used to study

probiotic adhesion. Clones of the HT-29 and Caco-2 cell lines such as absorptive Caco-2BB2, Caco-2/TC7 cells and HT29-19A cells, and the mucin-secreting HT29-C1.16E cells have been established (Servin, 2004). These cell lines have been shown to undergo morphological and functional differentiation *in vitro*, a characteristic feature of mature enterocytes of the small intestine. Moreover, these cell line models form junctional complexes, and so constitute a monolayer that mimics the intestinal epithelial barrier (Cereijido et al., 1998). Chauviere et al. (1992a) observed that bacterial adhesiveness is a strain-specific property. He found that among twenty-five strains of lactobacilli, seven adhered to enterocyte-like Caco-2 cells, whereas only three of them, including *L. acidophilus*, possessed calcium-independent adhesiveness.

The *L. acidophilus* LA 1 strain exhibited a high calcium independent adhesion onto human enterocyte like Caco-2 cells (Figure 2.3.2) and also bound strongly to the mucus secreted by the homogeneous cultured human goblet cell line HT29-MTX (Bernet et al., 1994). Heat-killed *L. acidophilus* LB was also found to adhere to Caco-2 and HT-29 cell line models (Coconnier et al., 1993). Several *Bifidobacterium* spp., including *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. infantis*, *B. longum*, *B. pseudocatenulatum*, *B. breve* 4, *B. infantis* 1 and *B. lactis* DR10 also adhered to Caco-2 and HT29-MTX cells (Gopal et al., 2001). Furthermore, the *in vitro* adhesion index system (Table 2.3.4) has proven to be extremely sensitive to factors such as pH, the presence of calcium ions, the number of lactobacilli, the presence of culture supernatant, and the growth phase in which the bacteria were harvested (Tuomola and Salminen, 1998; Blum and Reniero, 2000).

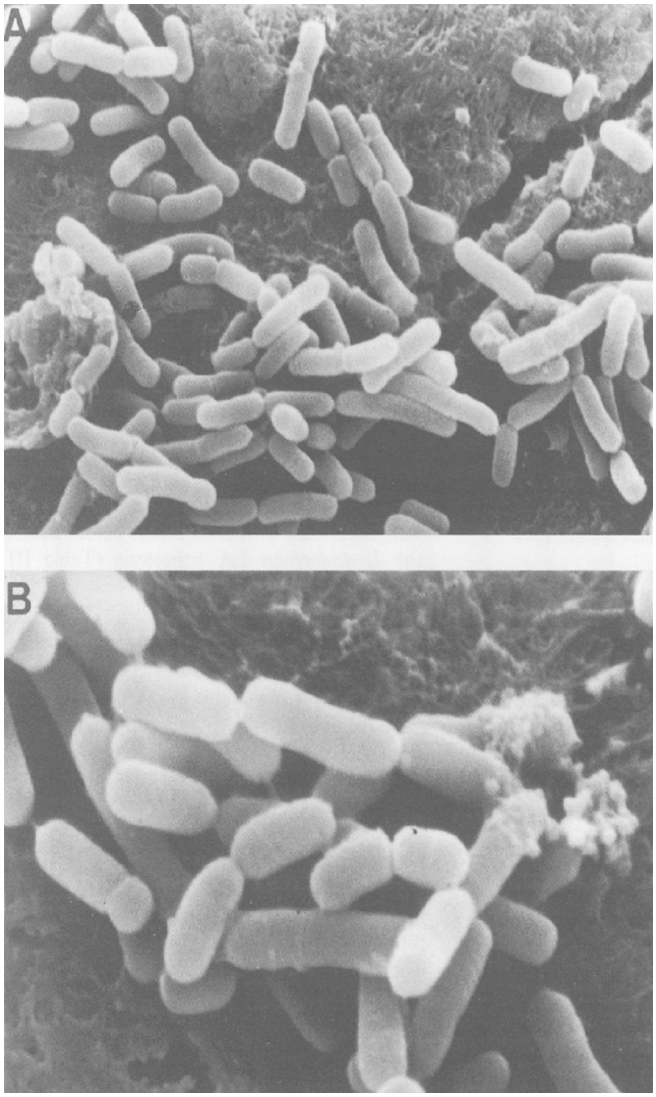

Figure 2.3.2 Examination by scanning electron microscopy of adherence of *Lactobacillus acidophilus* strain 1 onto the differentiated human intestinal epithelial cells Caco-2. (A) Low magnification of Caco-2 monolayer covered by *L. acidophilus* bacteria; (B) High magnification of *L. acidophilus* whole cells (Bernet et al., 1994)

Table 2.3.4 Lactobacilli with an adhesion index of at least one bacterium per Caco-2 cell (Adapted from Morelli, 2000)

Strain	Source	Adhesion index
L. acidophilus BG2FO4	Human	2.3
L. johnsonii LA1	Human	1.55
L. acidophilus LB	Human	2.1
L. rhamnosus GG	Human	1.25
L. acidophilus C7	Chicken	1.5
L. helveticus CNRZ239	Dairy	1.4
L. helveticus CNRZ 240	Dairy	2.1
L. delbrukii subsp. *lactis* CNRZ 239	Dairy	1.9
L. delbrukii subsp. lactis ATCC 7830	Unknown	2.3
L. delbrukii subsp. lactis LY	Yogurt	1.5

2.3.4 Antagonistic activities of probiotics against pathogens

LAB has a number of properties which make them highly suitable for probiotic therapeutics that are of pharmaceutical interest. Several mechanisms have been ascribed to probiotic action such as competitive exclusion, production of antimicrobial compounds, modulation of immune response, alternation of intestinal bacterial metabolic activity, alteration of microecology of the human intestine, and inhibition of bacterial translocation.

2.3.4.1 Competitive exclusion (CE)

CE can be defined as the principle that if two species try to occupy the same ecological niche, a superior species will eventually emerge to replace the inferior one (Vine et al., 2004). Various studies reported that adhesive probiotic bacteria can prevent the attachment of pathogens and remove them from the intestinal tract (Benno and Mitsuoka, 1992; Vine et al., 2004). CE of uropathogenic *Enterococcus faecalis* by *Lactobacillus* isolates were reported by Velraeds et al. (1996) in their *in vivo* studies done in a rat model of urinary infection. Heat-killed *L. acidophilis*

strain LB that adheres to the Caco-2 cell lines, was shown to inhibit the adhesion of diarrheagenic *E. coli* in a concentration-dependent manner (Chauviere et al., 1992b). The mechanism of CE in this study was explained to involve steric hindrance. Two *Bifidobacterium* strains including *B. bifidum* M6 and *B. bifidum* A1 were assessed for their ability to inhibit the adhesion and the displacement of enteropathogens including *Clostridium difficile* ATCC 9689, *Enterobacter sakazakii* ATCC 29544, *Salmonella enterica* serovar *typhimurium* ATCC 29631, *Escherichia coli* NCTC 8603 and *Listeria monocytogenes* ATCC 15313 (Gueimonde et al., 2007). The levels of displacement varied between 15 and 70% depending on the strains used. Higher affinity of *Bifidobacterium* strains for the receptors present in the mucus was explained as a mechanism of pathogen displacement in this study.

2.3.4.2 Co-aggregation of probiotics

Aggregation between microorganisms of the same strain (auto-aggregation), or between different species and strains (co-aggregation) for example with pathogens, as well as their ability to displace pathogens is an important property of probiotic organisms and may have greater advantage over non-co-aggregating organisms which are easily removed from GIT environment. The interaction of probiotic organisms with the natural gut flora is key to the potential success of the organism in terms of colonization and long-term persistence. Co-aggregation of probiotic bacterial strains has been suggested to enable them to form a physical-chemical barrier that prevents colonization by pathogenic bacteria (Collado et al., 2007b). Lactobacilli have been found to co-aggregate with some uropathogenic bacteria and inhibit their growth (Redondo-Lopez et al. 1990). Co-aggregation of *Lactobacillus acidophilus, Lactobacillus gasseri,* and *Lactobacillus jensenii* with pathogens like *Candida albicans, E. coli,* and *Gardnerella vaginalis* was observed by Boris et al. (1998). Furthermore, self-aggregation or clumping may

substantially increase the colonization potential of lactobacilli in environments with short residence times such as GIT. It has been hypothesized that combinations of probiotic bacterial strains may improve the health benefits compared to the strains alone (Collado et al., 2007b).

2.3.4.3 Probiotics modulating the immune response

The GIT is a complex ecosystem which contains up to 1×10^{14} cfu of bacterial cells of various phenotypes lining the epithelial wall and expressing complex metabolic activities (Zboril, 2002). The mechanism(s) of the immune response of the intestinal microbiota have been explained (Nicaise et al., 1999) by examining the regulation of interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α) and IL-12 production in macrophages from germ-free and from flora-associated mice, and germ-free mice colonized with *E. coli* and found that IL-12 production in the spleen was enhanced by intestinal flora. Interleukins are implicated in determining the relative levels of T-helper 1 (Th1) and T-helper 2 (Th2) responses, and play an important role in defending the host against intracellular microorganisms. Immune regulation involves homeostasis between Th1 and Th2 activity, with Th1 cells driving the type-1 pathway (cellular immunity) and Th2 cells driving the type-2 pathway (humoral immunity). The most important function of the resident intestinal microbiota is to act as a microbial barrier against pathogens by influencing humoral and cellular mucosal immune responses during the neonatal phase of life, and thereafter to maintain a physiologically-normal steady-state condition of inflammation throughout life (Cebra, 1999). In innate mucosal immunity, the host defense mechanisms are triggered as a result of specific recognition of pathogen-associated molecular patterns (PAMPs). Whereas, all endogenous bacterial species of the microbiota share microbe-associated molecular patterns (MAMPs). Epithelial and monocytic cells can sense the

environment of the GIT by means of pattern-recognition receptors (PRR) (Didierlaurent et al., 2002).

2.3.4.4 Production of antimicrobial compounds by probiotics

Several antimicrobial substances have been found to be produced by LAB that have considerable advantages in competition with pathogens and other harmful bacteria (Soomro et al., 2002). These substances include fatty acids, organic acids, hydrogen peroxide, and diacetyl, acetoin and the best studied small, heat-stable inhibitory peptides called 'bacteriocins' (Simova et al., 2009). The term bacteriocin was first introduced by Jacobs and coworkers in 1953, and defined as protein antibiotics of relative high molecular weight mainly working against the same, or closely related, species by adsorption to receptors on the target cells (Salminen and Wright, 1998a). Bacteriocins produced by LAB were divided into three classes: 1) lantibiotics; 2) small hydrophobic heat-stable peptides, and 3) large heat-labile proteins (Drider et al., 2006; see table 2.3.5).

The most common *Lactobacillus* spp. known to produce bacteriocins are *Lactobacillus sakei* and *Lactobacillus curvatus*. *Lactobacillus sakei* has been shown to possess antimicrobial activity against *Listeria monocytogenes* due to the production of the bacteriocins sakacin A, M, P, 674, K, and T (Schillinger and Lucke, 1989). The only purified bacteriocin approved for use in products for human consumption is nisin, which is produced by *Lactococcus lactis* subsp. *lactis* strains (Jack et al., 1995). *Escherichia coli* participates in antibacterial defense by producing colicins that function by forming pores in the cell membrane rendering the cells permeable to nuclease activity (Riley and Wertz, 2002).

Table 2.3.5 Classification of LAB bacteriocins (Adapted from Drider et al., 2006)

Class	Characteristics	Subcategory	Examples
I	Lantibiotics (containing lanthionine and β-lanthionine)	Type A (elongated molecules; molecular mass, < 4 kDa)	Nisin A Nisin Z Subtilin Epidermin
		Type B (globular molecules; molecular mass, 1.8 to 2.1 kDa)	Mersacidin Actagardin Mutacin II
II	Non-modified heat-stable bacteriocins containing peptides with molecular masses of <10 kDa	Subclass IIa (antilisterial pediocin-like bacteriocins)	Sakacin P, G, A etc.
		Subclass IIb (two-peptide bacteriocins)	Plantaricin EF Plantaricin JK
		Subclass IIc (other peptide bacteriocins)	Lactococcin 972
III	Protein bacteriocins with molecular masses of >30kDa		Helveticin J Millericin B

The antimicrobial action of bacteriocins involves increased permeability of the cytoplasmic membrane of the target cells for a broad range of monovalent cations, such as Na^+, K^+, Li^+, Cs^+, Rb^+ and choline, which leads to the destruction of proton motive force by dissipation of the transmembrane pH gradient and eventually to cell death (Oppegard et al., 2007; Simova et al., 2009). Bactericidal activity (Figure 2.3.3) of a bacteriocin-like substance produced by vaginal *Lactobacillus salivarius* subsp. *salivarius* CRL 1328 with activity against *Enterococcus faecalis*, *E. faecium*, and *Neisseria gonorrhoeae* has also been reported (Ocana et al., 1999).

Corr et al. (2007) demonstrated the *in vivo* antimicrobial effects of *L. salivarius* UCC118 against *L. monocytogenes* primarily depended on their capacity to produce the bacteriocin, Abp118. *Lactobacillus* spp. strain GG, isolated from the feces of a normal person, was reported to produce a bacteriocin with inhibitory activity in the pH range between 3 and 5 against a wide variety of bacterial species including Gram-positive and Gram-negative organisms (Silva et al., 1987).

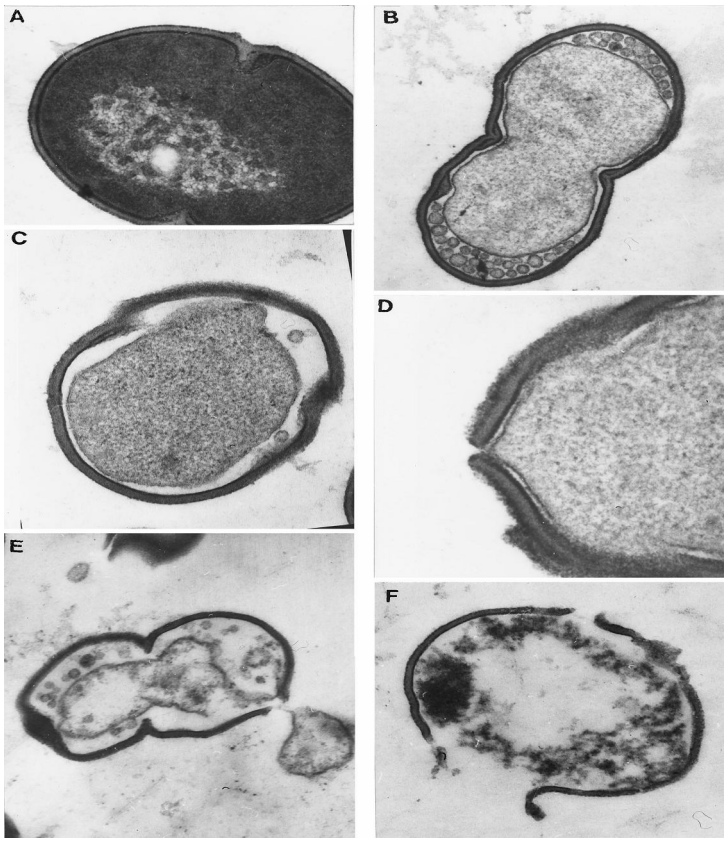

Figure 2.3.3 Electron photomicrographs of the effect of *L. salivarius* subsp. *salivarius* CRL 1328 bacteriocin-like substance on *E. faecalis*. The *Enterococcus* control cell (A), vesiculization of protoplasm (B), vesiculization of protoplasm and a damaged cell wall (C), pore formation in the cell wall (D), a disintegrated cell with loss of the protoplasmic material through a cell wall pore (E), and a disintegrated cell (F) (Ocana et al., 1999)

LAB produces lactic acid as the major metabolic end-product of sugar fermentation. Besides exerting its activity through lowering the pH and through its undissociated

form, lactic acid is also known to function as a permeabilizer of the Gram-negative bacterial outer membrane (Alakomi et al., 2000), allowing other compounds to act synergistically with lactic acid (Niku-Paavola et al., 1999).

The mode of action of organic acids on bacteria is explained in figure 2.3.4. Non-dissociated, or free, organic acids (RCOOH) can penetrate the bacterial cell wall, and thus enter the bacterial cytoplasm where they are exposed to a pH value near to neutrality (7.85 ± 0.05 for *E. coli*) and subsequently dissociate (Gauthier, 2002), releasing H^+ and anions (A^-). As a result of accumulating protons, the internal pH decreases. In case of non-pH sensitive bacteria (Figure 2.3.4) under low internal pH conditions, the organic acids remain in the free acid form and thus exit the bacteria. This process forms equilibrium between internal and external pH and protects the bacteria from detrimental effects of pH fluctuations (Lambert and Stratford, 1999). A specific mechanism (H^+ -ATPase pump) in pH-sensitive bacteria acts to bring the pH inside the bacteria back to a normal level, as they can not tolerate a large spread between the internal and the external pH. This process consumes energy and eventually stops the growth of the bacteria or even kills it. A lower cytoplasmic pH inhibits glycolysis, prevents active transport, and also interferes with signal transduction (Lambert and Stratford, 1999). Furthermore, the anionic (A^-) part of the acid cannot diffuse freely through the cell wall, and thus accumulates inside the bacterial cell. Accumulation of anions leads to internal osmotic problems for the bacteria.

Complete inhibition of *S. typhimurium* SL1344 growth was observed due to a pH-lowering effect produced by *L. acidophilus* LAP5 strain (Fayol-Messaoudi et al., 2005). Makras et al. (2006) demonstrated an inhibitory effect of lactic acid produced by probiotic lactobacilli upon invasion of *Salmonella* into Caco-2/TC7 cells. Viability of *Salmonella* was

found to be decreased rapidly after 1 h (~2 logs) and dramatically after 3 h (~8 logs) in the presence of *L. rhamnosus* GG supernatant containing lactic acid (De Keersmaecker et al., 2006).

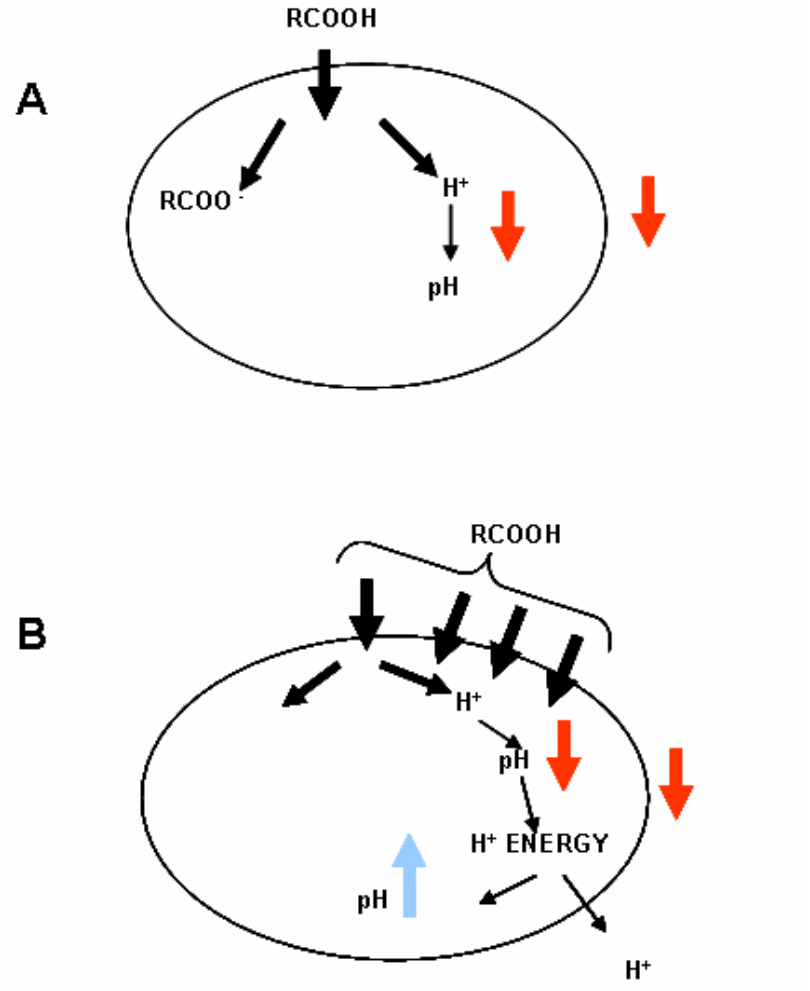

Figure 2.3.4 Mode of action of organic acids (RCOOH) on, A) non pH-sensitive bacteria (Lactic acid bacteria, Bifidobacteria), B) pH-sensitive bacteria (*Coliforms, Clostridia, Salmonella, Listeria* spp.) (Adapted from Gauthier, 2002)

Some LAB produce hydrogen peroxide (H_2O_2) under aerobic growth conditions and because of the lack of cellular catalase, pseudocatalase, or peroxidase, they release it into the environment to protect themselves from its antimicrobial action. It is a strong oxidizing agent and can oxidize the –SH group of membrane proteins of Gram-negative bacteria, which are especially susceptible (Ray, 2004). *Lactobacillus gasseri* CRL1421 and *L. gasseri* CRL1412

were found to produce H_2O_2 and lactic acid i*n vitro*, which reportedly reduced the viability of *Staphylococcus aureus* by 4 log units ($P < 0.05$), after 6 h of incubation (Otero and Macias, 2006).

2.3.4.5 Acylated homoserine lactones

Quorum sensing (QS) is a mechanism by which bacteria organize the expression of certain genes in response to their population density by producing, detecting and releasing small signal molecules. Quorum sensing was first observed in the marine bacterium, *Vibrio fischeri* (Nealson et al., 1970), an organism which undergoes bioluminescence under high cell densities. It has been demonstrated that inactivation of the QS system in pathogens can result in a significant decrease in virulence factor expression (Swift et al., 1997). Disruption of QS has become a new anti-infective strategy (Defoirdt et al., 2004). Acylated homoserine lactones **(**AHLs) are important intercellular signalling molecules used by many bacteria to monitor their population density in QS control of gene expression. These signalling compounds are the biochemical products of the LuxI family of proteins. AHLs acts as QS signal molecules in Gram-negative bacteria (Miller and Bassler, 2001) and small (oligo) peptides play this role in Gram-positive bacteria (Defoirdt et al., 2004). The family of autoinducers known as AI-2 operate in both Gram-negative and Gram-positive bacteria. Recently, AI-2-like molecules have also been detected in the cell-free culture fluid of several LABs, including *Lactobacillus rhamnosus* GG (Dirix et al., 2004). Besides the autoinduction, the most common mechanism of QS in Gram-positive bacteria consists of an ATP-binding cassette (ABC) transporter for secretion of the peptide, and a two-component system for sensing of the autoinducer concentration. Whereas, QS in Gram-positive *Staphylococcus aureus* and *Eenterococcus faecalis* are the exceptions of the ABC mechanism, which use a non-ABC transporter to secrete the

autoinducer. The QS system in *Bacillus subtilis* further involves genetic competence and sporulation, in which the peptide, after a Sec-dependent secretion and extracellular processing, is imported into the cytoplasm by an oligopeptide transport system where it interacts with intracellular receptors (Dirix et al., 2004). Studies done by McClean et al. (1997) showed that QS pigment production in Gram-negative *Chromobacterium violaceum* was stimulated by naturally-occurring AHL molecules. The acyl side chain has been found to be linked to the stimulatory and inhibitory effect of the AHLs. AHLs with an acyl side chain containing up to 8 carbon atoms were found to have a stimulatory effect and acted as QS agonists; whereas, acyl side chains containing 10 or more carbon atoms showed an inhibitory effect and acted as QS antagonists. A study done by Dong et al. (2002) suggested that enzymatic AHL inactivation could be used as a biocontrol strategy. In this study, expression of the AiiA (AHL-inactivating activity) enzyme in transformed *Erwinia carotovora* decreased the production of cell wall-degrading enzymes by the pathogen to about 10% and inhibited soft rot disease symptoms in susceptible plants almost completely.

A probiotic isolate, *Lactobacillus fermentum* RC-14, was reported to inhibit acute *Staphylococcus aureus* infection (Gan et al., 2002). The probiotic was found to secrete cell-surface extracellular matrix-binding proteins and a biosurfactant which prevented pathogen adherence to surgical implants. These findings illustrate different potential microbial antagonistic mechanisms other than antibiotic production, such as signal interference, for the control and prevention of biofilm formation by pathogenic bacteria.

2.4 Clinical evidence of probiotic efficacy

Metchnikoff (1907) was perhaps the first researcher to propose that fermented dairy products have beneficial properties. Various nutritional and therapeutic benefits (Table 2.4.1) to

the consumer were found to be associated with the consumption of lactic cultures and their fermented products (Danone, 2001; Parvez et al., 2006). The disorders hypolactasia, lactose malabsorption or maldigestion, and lactose intolerance, are characterized by a low concentration of the lactose cleaving enzyme lactase, or β-galactosidase, in the brush-border membrane of small intestinal enterocytes.

Table 2.4.1 Various special therapeutic or prophylactic properties of specific probiotics (Adapted from Parvez et al., 2006)

Microflora	Associated actions
Bifidobacteria spp.	Reduced incidence of enterocolotis
Enterococcus faecium	Decreased duration of acute diarrhoea
Lactobacillus strains	Efective in ameliorating pouchitis, decreased diarrhoea and symptoms of intolerence in lactose intolerent individuals, improved mucosal immune function, mucin secretion and prevention of disease
L. acidophilus	Significant decrease of diarrhoea in patients receiving pelvic irradiation, prevented uropatogens *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, lowered serum cholesterol evels
L. plantarum	Reduced incidence of diarrhoea and inflammation in inflammatory bowel, reduced bloating, pain and flatulence in irritable bowel syndrome, positive effect on immunity in HIV positive children
L. reuteri	Shortened the duration of acute gastroenteritidis and diarrhoea
L. rhamnosus	Enhanced cellular immunity in healthy adults in controlled trial
L. salivarius	Suppressed and eradicated *Helicobacter pyroli* in vitro and in vivo
Bacteroides spp.	Increased bacterial urease activity in chronic arthritis
Saccharomyces boulardii	Reduced *Cl. difficile* diarrhoea and antibiotic associated diarrhoea, shortened the duration of acute gastroenteritis

A significant proportion of the human population has some degree of intolerance to lactose. With the exception of the population of Northern and Central Europe, approximately 85% of adults worldwide are lactose malabsorbers (Goktepe et al., 2006). The LAB has an

excellent capacity to metabolize lactose. Studies by Kolars et al. (1984) showed that, in lactase-deficient subjects, lactose was absorbed significantly better from yogurt than from milk. Two different mechanisms of action have been put forward to explain this finding. Yogurt and probiotic LAB contain high levels of lactase, which is released within the intestinal lumen when these bacteria are lysed by bile secretions. Lactase then acts on the ingested lactose, thus relieving maldigestion symptoms. The reduced intestinal transit time of yogurt might also allow slower digestion of lactose. Several reviews (Rolfe, 2000; Kopp-Hoolihan, 2001; de Vrese et al., 2001) have described that some probiotics could improve lactose digestion and eliminate the symptoms of intolerance. While mechanisms by which these probiotics exert their effects are not fully understood yet; they may involve modifying gut pH, expressing β-galactosidase, exerting positive effects on intestinal functions and colonic microbiota. In a systematic review by Levri et al. (2005), in contrast, it was concluded that probiotic supplementation in general did not alleviate the symptoms of lactose intolerance in adults. The lactose contained in yogurts can be considered as a prebiotic for people with lactose maldigestion (Szilagyi, 2004). Regular consumption of lactose influences their colonic microbiota and reduces lactose intolerance (Hertzler and Savaiano, 1996).

One particular strain, *Lactobacillus casei* DN-114001, has been reported to reduce diarrhoeal morbidity by 40% in children (Agarwal and Bhasin, 2002). Study on pregnant women and newborns suggested that consumption of probiotic *L. rhamnosus* GG reduced the rate of newborns having atopic dermatitis (Kalliomaki et al., 2001).

In an Australian study, 178 newborns of women with allergies who received either *L. acidophilus* LAVRI-A1 or placebo daily for the first 6 months of life showed no difference in atopic dermatitis (probiotic, 23/89 versus placebo, 20/88; P = 0.629). However, at 12 months, the

rate of sensitization was significantly higher in the probiotic group (P = 0.030). These results suggested that the probiotic treatment has increased the risk of subsequent cow's milk sensitization (P=0.012) (Taylor et al., 2007). Similarly, a double-blind, randomized, placebo-controlled trial study was conducted by Abrahamsson et al., (2007) on 188 subjects with allergic disease, in which the mothers received *Lactobacillus reuteri* ATCC 55730 daily from gestational week 36 until delivery, and their babies continued with the probiotic until 12 months. Probiotic supplemented babies showed less IgE-associated eczema during the second year.

2.5 **Prebiotics**

Association of colonic microflora with beneficial health effects is now well established. Consequently, the use of prebiotics as functional food ingredients to manipulate the composition of colonic microflora to improve health is currently a growing interest (Wang, 2009). According to Solange et al., (2007) prebiotics are non-digestible carbohydrates (oligosaccharides) of low molecular weight and intermediate in nature between simple sugars and polysaccharides. Resistant short-chain carbohydrates (SCCs) are also referred to as non-digestible oligosaccharides or low digestible carbohydrates (LDCs) (Cummings et al., 2001). Prebiotics are defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Wang, 2009). Prebiotics resist hydrolysis and absorption in the upper parts of the GIT and may selectively be metabolized by at least one type of probiotic microorganism in the colon, leading to growth and/or metabolic activation of the probiotic microorganisms, improvement of the colonic flora, and beneficial luminal or systemic effects for the health of the host (Gibson and Roberfroid, 1995). Bifidobacteria and lactobacilli are the most

important bacterial genera targeted for selective stimulation by prebiotics (Teitelbaum and Walker, 2002). The "bifidogenic" effects of SCC or LDCs make it possible for their inclusion into conventional food products (Holzapel et al., 1998). Oligosaccharides that have been considered as prebiotics include galacto-oligosaccharides, isomalto-oligosaccharides, soybean-oligosaccharides, lactosucrose, and xylo-oligosaccharides (Rastall and Gibson, 2002). For the comparison of prebiotic effects of dietary oligosaccharides, a quantitative prebiotic index (PI) equation has been developed by Palframan et al. (2003). This equation is based on the changes in key bacterial groups like bifidobacteria, lactobacilli, clostridia and bacteroides during fermentation. The changes in bacterial groups from previous studies were entered into the equation in order to determine a quantitative PI score. Table 2.5.1 shows examples of prebiotics used for human consumption (Wang, 2009).

Table 2.5.1 Examples of ingredients that are commonly regarded as human prebiotics (Wang, 2009)

Prebiotics
Inulin-type fructans
Trans-galactooligosaccharides (TOS)
Lactulose
Isomalto-oligosaccharides
Lactosucrose
Xylo-oligosaccharides
Soybean-oligosaccharides
Gluco-oligosaccharides
Fructo-oligosaccharides (FOS)
Galacto-oligosaccharides (GOS)

2.5.1 Inulin and fructo-oligosaccharides (FOSs) as prebiotics

Inulin and FOSs are considered as typical "bifidogenic factors" and are probably the most commonly-used prebiotics in the market (Bouhnik et al., 1999). Growth of *L. casei* was significantly ($P \leq 0.05$) higher when inulin of different chain lengths was incorporated at a concentration of 1.5 g/100 g yogurt mix (Aryana et al. 2007). Similarly a study by Akalin et al.

(2004) reported higher counts of *Bifidobacterium longum* (10^6 cfu/g) and *Bifidobacterium animalis* (3.59-2.25 × 10^7 cfu/g) in yogurts containing FOS during 28 days storage at 4°C; whereas, this level was maintained for only 7 days in yoghurt without any prebiotic. Scientific studies done in Japan showed that the consumption of FOS in the diet increases the population of bifidobacteria and other beneficial micro-organisms, even in the absence of probiotics (Losada and Olleros, 2002). Similarly, increased numbers of lactobacilli and bifidobacteria were found following the consumption of FOS in a study by Mitsuoka (1990). To assess the effects of lactulose administration on faecal bifidobacteria, a controlled, randomised, double-blind, and parallel group trial study was conducted on 16 healthy volunteers by Bouhnik et al. (2004). Participants ingested lactulose or placebo (sucrose) at a dose of 5 g twice a day for 6 weeks. Results showed that ingestion of 10 g of lactulose per day increased fecal bifidobacterial counts from day 0 to day 42 (8.25 ± 0.53 to 9.54 ± 0.28 log cfu/g wet weight, respectively). Shin et al. (2000) reported a decrease in mean doubling time and increase in viability of *Bifidobacterium* spp. (Bf -1 and Bf -6) in skim milk during 4 weeks of storage at 4°C, containing inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) at a concentration of 50 g/L. A 67% viability of Bf-1 and 45% viability of Bf-6 were retained in the presence of FOS. When grown in the presence of GOS, 52 and 39% retention of viability of Bf-1 and Bf-6 was observed, respectively. Inulin was the least effective ($P < 0.05$) in retaining viability of either strain.

2.5.2 Health benefits of prebiotics

A number of beneficial effects are associated with prebiotics, including the favorable influence on the small bowel by improved sugar digestion and absorption, glucose and lipid metabolism, and protection against known risk factors of cardiovascular diseases (Bruzzese et al., 2006) as shown in Table 2.5.2.

Table 2.5.2 Microbiological changes reported in human feeding studies with prebiotics (Adapted from Macfarlane et al., 2008)

Substrate	Type of study	Delivery	Effect on microbiota
GOS	Placebo controlled study involving 12 subjects	10 g prebiotic fed daily for 8 weeks	Faecal excretion of bifidobacteria and lactobacilli increased
	Feeding study with 12 subjects	2.5 g prebiotic given per day for 3 weeks	Increased bifidobacteria in faeces, reductions in numbers of clostridia and bacteroides
TOS	Feeding study with 8 volunteers	Subjects given 10 g TOS for 3 weeks	Significant increases in faecal bifidobacteria, enterobacteria unaffected
	Parallel study involving 40 healthy subjects	7.5 or 15 g prebiotic fed per day for 3 weeks	Lactobacilli increased in 15 g/day, no effects on clostridia, small reductions in enterobacteria
GOS/FOS	Placebo-controlled study with 90 term infants	Infant formula supplemented with either 4 or 8 g/l low molecular weight GOS and high molecular weight FOS for 28 days	Significantly increased lactobacilli and bifidobacteria in the two GOS feeding groups.
GOS	Placebo-controlled study involving 30 subjects	Volunteers given 8.1 g GOS syrup, 8.1 g GOS plus 3 X 10^{10} *Bifidobacterium lactis* Bb-12, or 3 X 10^{10} *B. lactis* without GOS for 3 weeks	Little change in faecal bifidobacteria seen with GOS alone. GOS plus B. lactis and *B. lactis* on its own resulted in faecal excretion of the organism and reduced numbers of *B. longum.*
GOS/FOS(9 :1 ratio)	Double-blinded, randomized controlled trial (DBRCT) involving 20 infants aged 28–90 days	Infant formula supplemented with 0.8 g /100 ml GOS/FOS for 6 weeks	Increased total bifidobacteria counts in stools. Reduced *B. adolescentis* compared to standard infant formula controls. Bifidobacteria spp. composition in prebiotic infants similar to that found in breast-fed babies, with *B. infantis, B. breve* and *B. longum* predominating
GOS/FOS (9:1 ratio)	Feeding study involving 42 preterm infants, 15 placebos, and a reference group given fortified mothers milk	Formula food supplemented with 10 g/l prebiotic mixture	Bifidobacteria numbers greatly increased from initially low levels, compared to un-supplemented controls. No significant effects on bacteroides, clostridia, enterobacteria or yeasts

Various *in vivo* and *in vitro* studies shows health benefits of prebiotics such as FOS and GOS alone, or in combination with inulin (Macfarlane et al., 2008). Production of short chain fatty acids (SCFAs) is considered to be a major beneficial feature related to the primary prevention of colorectal cancer (Scheppach et al., 2001). SCFAs and lactic acid are produced as a result of metabolism of FOS by fermentative bacteria, leading to a drop in the pH of the large intestine. Low pH conditions were reported to be ideal for growth of bifidogenic flora. A study done by Liu et al. (2004) on 55 cirrhotic patients, were randomized to receive a probiotic plus prebiotic (synbiotic) preparation containing 4 freeze-dried probiotics namely *Pediacoccus pentoseceus*, *Leuconostoc mesenteroides*, *L. paracasei* subsp. *paracasei* and *L. plantarum*. Each probiotic was given at a dose of 10^{10} cfu/sachet, along with 10 g of bioactive, fermentable fiber (beta glucan, 2.5 g; inulin, 2.5 g; pectin, 2.5 g; resistant starch, 2.5 g) (n = 20), fermentable fiber alone (n = 20), or placebo (n = 15) for 30 days. The controls had significant fecal overgrowth of potentially pathogenic *E. coli* and *Staphylococcus* spp., while the synbiotic treated subjects had significantly increased fecal content of *Lactobacillus* strains.

2.6 Viability of probiotic microorganisms

Fermented dairy products such as milk and yogurt are the most accepted food carriers for live probiotic delivery to the human GIT. A recommended intake of probiotics is 10^8-10^9 viable live cells daily (Lourens-Hattingh and Viljoen, 2001) in order to get the beneficial health effects. Standards have been made in many countries for the numbers of viable probiotic bacteria that are present in commercial fermented products. For example, in Japan, fermented milks and LAB containing beverages must contain a minimum of 10^7 cfu/mL or gram of product (Robinson, 1987). Despite the importance of viability of these beneficial bifidobacteria, surveys have shown poor viability of bifidobacteria in yoghurt preparations (Akalin et al., 2004). Several factors, like

acidity of the product, post acidification (acid produced during storage), level of oxygen in the products, sensitivity to antimicrobial substances produced by bacteria, temperature of storage during manufacture and storage of yoghurt, have been found to reduce the viability of probiotics (Lankaputhra and Shah, 1995; Dave and Shah, 1997). Thus, maintaining viability of bifidobacteria until the products are consumed in order to ensure the delivery of live organisms has been of much interest.

2.6.1 Influence of cryoprotectants

Freeze-drying is a preservation process that has been employed to improve the survival of probiotic microorganisms (Rybka and Kailaspathy, 1995). Survival and rapid growth after freezing is one of the most important characteristics for the selection of probiotics because they are most often delivered in the form of frozen dairy desserts. Addition of compatible cryoprotectants into the media or yogurt mix prior to fermentation has been shown to improve the viability of probiotic microorganisms (Kets et al., 1996). Improvement in the viability of probiotics may occur as the cryoprotectants inhibit the formation of intracellular or extracellular ice by binding to the water (Capela et al., 2006). The cryoprotectant Unipectine^{TM} RS 150 was found to improve the number of viable *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* (GG) by 1 log during storage in fermented dairy desserts (Shah and Ravula, 2000). In another study, an 80% improvement in the survival of *B. longum* 536 was found by using Unipectine^{TM} RS 150 (Capela et al., 2006).

2.6.2 Encapsulation

Encapsulation is a process whereby cells are retained within an wall material to reduce cell injury. Encapsulation in hydrocolloid beads has been investigated as a means to protect and

improve viability of probiotic microorganisms in food products and in the intestinal tract (Rao et al., 1989). Other benefits of encapsulation includes: protection of probiotics from bacteriophage (Steenson et al., 1987), increased survival during freeze-drying and freezing (Kim et al., 1995), and greater stability during storage (Kebary et al., 1998). Hou et al. (2003) demonstrated that encapsulation of *Lactobacillus delbrueckii* spp. *bulgaricus* increased their bile tolerance, and viability was elevated by approximately four log units after encapsulation within artificial sesame oil emulsions. Capela et al. (2006) found improved viability of probiotic organisms encapsulated in 3% v/w sodium alginate in freeze-dried yogurt after 6 months of storage at 4 (~8 log cfu/g) and 21°C (~6 log cfu/g). Spray drying, freeze drying, fluidized bed drying are techniques which are used for converting probiotic cultures into a concentrated powdered form. However, these techniques do not protect the bacteria from the product environment or during their passage through the GIT because they release the bacteria completely into the product. The viability of *Bifidobacterium bifidum* BB-12 and *L. acidophilus* LA-5 encapsulated in Na-alginate by either an extrusion or an emulsion technique and used in white-brined cheese was monitored (Ozer et al., 2009). Both encapsulation techniques were found to be effective in keeping the numbers of probiotic bacteria higher than the level of the therapeutic minimum ($>10^7$ cfu/g). While the counts of non-encapsulated probiotic bacteria decreased approximately by 3 logs, the decrease was more limited in the cheeses containing microencapsulated cells (approximately 1 log).

Depending on the method used to form the beads, the encapsulation techniques applied for probiotics for use in fermented milk products or biomass production can be classified as either the: i) extrusion or droplet method, and ii) emulsion or two phase systems (Figure 2.6.1).

Both techniques have been found to increase the survival of probiotic bacteria by up to 80-95% (Audet et al., 1988; Rao et al. 1989).

2.6.2.1 Extrusion technique

In extrusion encapsulation, microorganisms are added into a hydrocolloid solution (alginate) and then the cell suspension is extruded through a syringe needle to form droplets, which free-fall into a hardening solution or setting bath (Figure 2.6.1).

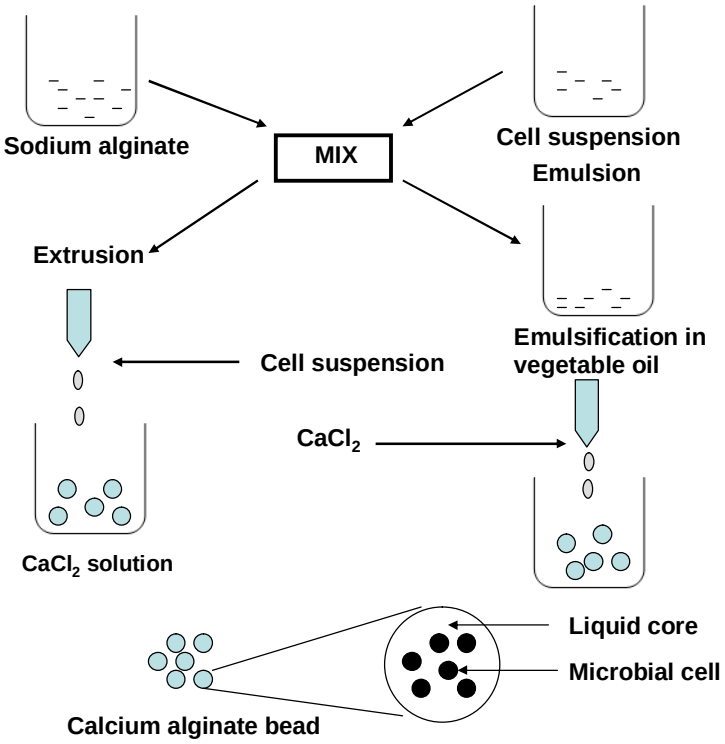

Figure 2.6.1 Flow diagram of encapsulation of bacteria by the extrusion and emulsion techniques (Reproduced from Krasaekoopt et al., 2003)

The size and shape of the beads depends on the diameter of the needle and the distance of free-fall (Kim, 1995). Alginate is used commonly as supporting material in this technique. The concentrations of alginate used to form the gel varies from a very low concentration of 0.6% to

form a gel with 0.3M $CaCl_2$ to 1–2% alginate and 0.05–1.5M $CaCl_2$ (Krasaekoopt et al., 2003). The size and sphericity of the bead also depends on the viscosity of the sodium alginate solution, the distance between the syringe and the calcium chloride collecting solution and the extruder orifice diameter. As the concentration of sodium alginate increases, the size of the beads decreases. Using a 0.27 mm syringe resulted in a bead size of 2–3 mm. The composition of the alginate also influences bead size; small beads result from ‘‘low guluronic’’ alginates (Krasaekoopt et al., 2003).

2.6.2.2 Emulsion technique

In the emulsion technique, a small volume of the cell-polymer suspension (the discontinuous phase) is added to a large volume of a vegetable oil (e.g. canola or corn oil). The mixture is homogenized to form a water-in-oil emulsion. Once formed, the water-soluble polymer must be insolubilized (cross-linked) to form tiny gel particles within the oil phase (Figure 2.6.1). The smaller the internal phase particle size of the emulsion, the smaller the final microparticles will be. The supporting material used in this technique may include κ-carageenan and locust bean gum (Audet et al., 1988), cellulose acetate phthalate (Rao et al., 1989), alginate (Sheu and Marshall, 1991), chitosan and gelatin (Groboillot et al., 1993).

2.7 Synbiots

The combination of a probiotic and prebiotic is referred to as a synbiot. Synbiots have been shown to confer health benefits beyond those of either on its own. Rowland et al. (1998) showed the enhanced reduction in the number of colonic aberrant crypt foci, as well as colon carcinogenesis in rats (Gallaher and Khil, 1999). Antibiotic-associated diarrhea was found to be prevented by the combined application of *Lactobacillus sporogenes* and FOS in children (Rosa

et al., 2003). Synbiotic combination of *L. paracasei* and maltodextrin was found to decrease the colonization of *E. coli* in the jejunum of piglets. This symbiotic combination led to an increase in lactobacilli and bifidobacteria and decreased clostridia and enterobacteria (Bomba et al., 2002). On the other hand, Anderson et al. (2004) did not find any improvement of gut barrier function in surgical patients following synbiotic therapy.

Therefore, further investigation is needed to continue, and extend our understanding of probiotics, along with the efficacy once encapsulated. The present research investigates the ability of probiotic bacteria to survive in simulated gastric conditions of the GIT, as well as their ability to become established on the surface of the intestinal epithelium or to other resident microflora. An attempt was made to determine the antimicrobial potential of the probiotic bacteria against a panel of selected enteric pathogens. The probiotic bacteria were also characterized using biochemical techniques to aid in their identification during follow-up research. Model HCl/bile-based model gastric systems were then be used to compare the effect of encapsulation on the survival of the probiotic bacteria.

3 In vitro characterization of probiotic survival, adherence and antimicrobial resistance: candidate selection for encapsulation in a pea protein isolate-alginate delivery system

Abstract

A panel of probiotic strains, including *Lactobacillus acidophilus* ATCC 11975, *Bifidobacterium infantis* ATCC 15697D, *B. catenulatum* ATCC 27675 and *B. adolescentis* ATCC 15703, were screened for a number of traits, including their resistance to acid and bile and adherence to Caco-2 cells. Under simulated gastric conditions (pH 2.0), *L. acidophilus* was the most acid-tolerant strain (*D*-value 10.2 ± 0.8 min), and was able to survive for 30 min; whereas, the other probiotics tested underwent a rapid (within the first 5 min at pH 2.0) 4-5 log cfu/mL loss in viability. Notably, the number of viable *L. acidophilus* decreased by less than 1 log cfu/mL over the same interval. All probiotics tested were able to survive 5 h of exposure to 0.3% (w/v) Oxgall bile at pH 5.8. The relative ranking of probiotic adherence to Caco-2 cells was determined to be: *L. acidophilus* > *B. catenulatum* > *B. adolescentis* > *B. infantis,* which correlated with 4.5×10^4, 3.1×10^3, 2.6×10^1, and 1.5×10^1 cfu/mL associated with Caco-2 cell monolayers, respectively. The probiotics also revealed varying degrees of resistance for the antimicrobial agents: ciprofloxacin, naladixic acid, kanamycin and sulfisoxazone. Determination of carbon source utilization patterns indicated that all strains grew to the highest culture optical densities on D-xylose. *L. acidophilus* was determined to be the best overall *in vitro* performer, and hence was selected for encapsulation within a ~3 mm diameter pea protein-alginate matrix. Upon exposure of encapsulated *L. acidophilus* to simulated gastric conditions (pH 2.0) there was only a ~1 log cfu/mL loss of cell viability observed over a 2 h period; whereas, unprotected *L. acidophilus* cells experienced a reduction in cell viability of greater than 6 log cfu/mL over the same period. The present study indicates that the encapsulated delivery of probiotics is a feasible means for enhancing probiotic survival during passage through the upper gastrointestinal tract.

3.1 Introduction

Probiotics are microorganisms that are consumed orally and that reach the GIT in a viable state, where they subsequently contribute to the beneficial activity of intestinal microflora and, thus, to the health of its host. Most probiotic bacteria are LAB and among them lactobacilli and bifidobacteria reportedly play a significant role in maintaining the intestinal microbial ecosystem and in stimulating the immune system of the host (Saarela et al., 2002). Finegold et al. (1977) demonstrated that while lactobacilli were not the predominant species among the intestinal microflora, they were commonly isolated from the GITs of healthy humans. Others have shown that typical numbers of endogenous faecal lactobacilli per gram of faeces ranged from 10^5 to 10^8 cfu (Kimura et al., 1997; Holzapfel et al., 1998).

It has been shown that the functional and technical properties of probiotics are strain-specific, and thus it is necessary to screen various isolates for a variety of *in vitro* properties to obtain new probiotic candidates is of utmost important (Collins et al., 1998; Ouwehand et al., 2002). These include general aspects (e.g., origin, identity, resistance to mutations, resistance to environmental stress prevailing in the GIT such as low pH, bile acid and pancreatic juice), technical considerations (e.g., *in vitro* growth and processing properties), functional and beneficial properties (e.g., adhesion and colonization to intestinal mucosa, competitiveness, antagonism against pathogens, and stimulation to immune response) and safety concerns (e.g., no invasive potential, no transferable resistance against therapeutic antibiotics and no virulence factors) (Holzapfel et al., 2002). Lactobacilli fulfill these criteria (Dunne et al., 2001) and their use is now being supported by clinical benefits (Reid et al., 2003). Cellular stress on probiotics begins in the stomach, where pH values as low as 1.5 are typically encountered (Lankaputhra and Shah, 1995). Following passage through the stomach, probiotics come in contact with bile in the upper intestinal tract, the concentration of which is variable and difficult to predict at any

given time (Northfield and McColl, 1973). After traveling through these relatively harsh conditions, the probiotic should ideally adhere to the lower intestinal tract epithelia and microflora where it would proliferate as a stable member (Conway et al., 1987), so that they are able to exert health benefits to the host for prolonged periods. It is noteworthy that some conjecture presently exists as to whether all probiotics must actually colonize the epithelial membranes of the intestine for a probiotic effect to be realized.

While there continues to be a lack of clear information to the biologically-effective probiotic dose, it is clear that increasing the number of viable probiotic that reach the upper GIT is important. Improved survival of probiotics exposed to the low pH gastric environment (Rao et al., 1989; Wenrong and Griffiths, 2000) and bile salts (Lee and Heo, 2000), molecular oxygen in the case of obligatory anaerobic microorganisms, bacteriophages (Steenson et al., 1987), chemical antimicrobials (Sultana et al., 2000), and increased survival during freezing and freeze drying (Shah and Rarula, 2000) have all been reported following probiotic microencapsulation. Additional advantages of probiotic encapsulation over free cells include an increase of sensory properties, stability (Gomes and Malcata, 1999), and immobilization of the cells for their homogeneous distribution throughout the product (Krasaekoopt et al., 2003). A variety of substrates have been employed as encapsulating materials for entrapping live microorganisms, including alginate, whey protein, pea protein, gelatin, methylcellulose, etc. (Sheu et al., 1993; Prevost and Divies, 1988; Gombotz and Wee, 1998; Pierucci et al., 2007). Among these, alginate-gel beads have frequently been used for the immobilization of LAB (Sheu et al., 1993), as it is an accepted food additive and has the benefit of being nontoxic to the cells being immobilized (Prevost and Divies, 1988).

The objective of present study was to conduct *in vitro* screening of a panel of probiotic organisms, using acid resistance, bile toxicity and ability to attach to the human intestinal cells, as test variables. Probiotics were also screened for resistance to commercial antibiotics and their ability to utilize different sole carbon sources. Based on the above tests, a candidate probiotic was selected for encapsulation in a pea protein-alginate matrix and then challenged with simulated gastric conditions.

3.2 Materials and methods

3.2.1 Bacteria and culture conditions

The probiotic strains *Lactobacillus acidophilus* ATCC 11975, *Bifidobacterium infantis* ATCC 15697D, *B. catenulatum* ATCC 27675 and *B. adolescentis* ATCC 15703 were stored at -80ºC using 10% glycerol amended in culture broth as a cryoprotectant. DeMan Rogosa and Sharpe (MRS) broth (VWR, Mississauga, ON, Canada) was used as a media for routine cultivation of lactobacilli; whereas, MRS supplemented with 0.05% (w/v) L-cysteine HCl was used for cultivation of bifidobacteria. Prior to each experiment, stock cultures were propagated twice in sterile broth under anaerobic conditions (10% H_2, 10% CO_2, and 80% N_2) at 37°C for 24 h to provide inocula for subsequent experiments. Inocula of each strain were harvested at the early stationary phase of growth (~ 20 h; data not shown) by centrifugation at 3,250 × g (Model 5810 R with swing-bucket rotor A-4-81; Eppendorf, Hamburg, Germany) for 10 min at 4°C. Cells were then washed twice with phosphate-buffered saline (PBS; 4.3 mM $Na_2HPO_4.7H_20$, 137.0 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.2) and resuspended in peptone saline (PS; 8.5 g/l NaCl, 1.0 g/l peptone, pH 7.0).

3.2.2 Probiotic resistance to simulated gastric juice (SGJ)

Probiotic resistance to SGJ was performed according to the following experimental design; washed cell suspensions (~10^5 cfu/mL) were inoculated in triplicate into SGJ without pepsin (0.08 M HCl containing 0.2% w/v NaCl) with pH adjusted to 2.0 and 6.0 (control) using either HCl or NaOH. Samples were mixed well, incubated at 37°C and aliquots removed every 5 min for 2 h and serially diluted in PS. Surviving bacteria were enumerated by pour plating. *D*-values for each probiotic were calculated for the 0 to 5 min. SGJ exposure period using the equation, $D = t / n$ where $n = (\log N_0 - \log N_t) = 1 \log_{10}$ reduction of the cell number, D = Decimal reduction time (min) at pH 2.0, N_0 = bacteria at 0 time; N_t = surviving bacteria after an exposure time, t (min). The best surviving probiotic at pH 2.0 will be tested for its survival at pH 4.0 and 5.0.

3.2.3 Probiotic resistance to bile salts

The method of Gilliland et al. (1984) was used to evaluate the growth potential of the four probiotic strains in broth media with and without Oxgall bile salts (Sigma-Aldrich Inc., St. Louis, MO, USA). Accordingly, MRS broth without (control) and with 0.3% bile (w/v) was inoculated with 100 µL (~10^6 cfu/mL) of each overnight culture, and then incubated under anaerobic conditions for 24 h at 37ºC and 100 rpm shaking. Growth was monitored hourly by measuring absorbance at 600 nm using a spectrophotometer. Experiments were conducted in triplicate.

Survival of the different probiotic strains in the presence of 0.3% Oxgall was monitored by the standard plate count method. Overnight cultures were inoculated into flasks containing sterile MRS (control) and MRS-bile, and incubated at 37°C for 5 h, anaerobically. Aliquots were

removed every hour and serially diluted in PS. Surviving bacteria were enumerated by spread plating.

3.2.3.1 Microscopy

An inverted light microscope (Nikon Eclipse, TS100; 400 X NA 0.55 lens) was used to take photographs of crystals formed by probiotic bacteria in the MRS broth containing 0.3% bile and MRS broth without any added bile (control).

3.2.4 Probiotic adherence to Caco-2 cell lines

3.2.4.1 Cell culture

Enterocyte-like Caco-2 cells were kindly provided by Dr. Wolfgang Köster (Vaccine Infectious Disease Organization (VIDO), Saskatoon, Canada). Caco-2 cells were routinely cultured at 37ºC in 5% CO_2-95% air atmosphere in Dulbecco Modified Eagle's Minimal Essential Medium (DMEM; Aldrich Sigma, ON) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Canada Inc., Burlington, ON). Caco-2 cell monolayers were used for the probiotic adherence assay at the post-confluence stage (after 10 d). The concentration of Caco-2 cells in the monolayer was determined by trypsinizing the cells using Trypsin-EDTA for 10 min at 37ºC followed by counting in a hemocytometer. Caco-2 cells (50 to 52 passages) were seeded at a concentration of 3×10^4 cells/mL in sterile 24-well tissue culture plates. The culture medium was changed every 2 d.

3.2.4.2 Adhesion assay

Adhesion assay was conducted as outlined by Jacobsen et al. (1999) and Pennacchia et al. (2006), as briefly outlined below. Post-confluence Caco-2 monolayers were gently washed two times with Hank's Balanced Salt (HBS) solution (Invitrogen Canada Inc., Burlington, ON).

Probiotic bacteria in their early stationary phase of growth (~20 h; data not shown) were harvested and washed twice with PBS by centrifugation at 3,250 × g (Model 5810 R equipped with a swing-bucket rotor A-4-81; Eppendorf) for 10 min at 4°C and diluted (10x) with DMEM to provide a bacterial concentration of ~5×10^5 cfu/mL. A 2 mL aliquot of each bacterial culture was used to inoculate the tissue culture plate wells containing Caco-2 cells, followed by incubation at 37ºC in 5% CO_2-95% air atmosphere for 2 h. Control wells contained un-inoculated DMEM. The viability of probiotic strains was also monitored in 5% CO_2-95% air atmosphere for 2 h. Following incubation, Caco-2 cell monolayers were washed 5 times with HBS solution to release unbound bacteria. Washed Caco-2 cell monolayers were trypsinized using Trypsin-EDTA for 5 min at 37°C to detach the bacterial cells from Caco-2 cells. The number of viable adhering bacteria was determined by spread plating serial 10-fold dilutions on MRS agar and incubating at 37°C for 48 h under anaerobic conditions.

3.2.5 Probiotic susceptibility to antibiotics

Antibiotic resistance of each probiotic strain was determined using the Sensititre CMV1AGNF plates (Trek Diagnostic Systems, Inc., Westlake, OH, USA) that contained 17 antimicrobial agents incorporated in dehydrated form into the wells of a plastic microtiter plate in either single or paired concentrations, as specified by the NARMS (National Antimicrobial Resistance Monitoring System) protocol of the CDC. Inocula for the antibiotic sensitivity assays was prepared by suspending isolated colonies of each probiotic (picked from fresh cultures on MRS agar plates incubated anaerobically for 48 h at 37°C) in a tube with 5 mL of sterile distilled water to an optical density of 0.5 McFarland standard, and subsequently diluting 1:100 in MRS broth. A 50 µL aliquot of this MRS broth suspension (containing ~10^6 cfu/mL) was then transferred to each microtiter plate well. The plates were sealed and incubated

anaerobically at 37°C for 48 h (Klare et al., 2005; Doern et al., 1985), and then examined manually for the presence of turbidity. A 'susceptibility category' test result was assigned to each antimicrobial, as outlined below. With antimicrobials present in paired concentrations, turbidity in both wells was considered 'resistant', turbidity only in the well containing the lower concentration was considered 'intermediate', and absence of turbidity in either well was considered 'susceptible'. For antimicrobials present in individual concentrations, turbidity was considered 'resistant' and absence of turbidity was considered 'susceptible'. The minimum inhibitory concentration (MIC) breakpoints were determined according to the national committee for clinical laboratory standards (NCCLS, 2002a; and NCCLS, 2002b).

3.2.6 Probiotic carbon source utilization profiles

Biolog Ecoplates (Biolog, Inc., Hayward, CA, USA) contain 31 different sole carbon sources and can be used to screen substrate preference amongst different pure cultures. Cultures grown on solid MRS agar media for 48 h were swabbed and resuspended in sterile distilled water. The turbidity of these culture suspensions were then compared with the Biolog GP microplate turbidity standard (~10^8 cfu/mL). Biolog Ecoplate wells were subsequently inoculated with 150 µl of each cell suspension. Following an initial (time 0) reading, Biolog plates were incubated anaerobically at 37ºC for 48 h. The substrate utilization patterns were analyzed with a microplate reader at a wavelength of 590 nm.

3.2.7 Growth experiments

Test was conducted according to Vernazza et al., 2006. Basal medium was prepared as follows: in g/L peptone water (Sigma chemicals company, Poole, UK) 2.0, yeast extract (Oxoid Ltd, Hampshire, UK) 2.0, NaCl 0.1, K_2HPO_4 (Oxoid Ltd, Hampshire, UK) 0.04, KH_2PO_4 (Oxoid

Ltd, Hampshire, UK) 0.04, $MgSO_4.7H_2O$ 0.01, $CaCl_2$ $2H_2O$ 0.01, $NaHCO_3$ 2.0, Tween 80 (BDH chemicals, Poole, UK) 2.0 mL/L, hemin (Sigma chemicals company, Poole, UK) 0.005, vitamin K1 10 μL/L, Cysteine-HCl 0.5, bile salts (Oxoid Ltd, Hampshire, UK) 0.5, pH 7.0. Test carbohydrate solutions including D-lactose (BDH chemicals, Poole, UK), methyl-D-glucoside (NB Co. Cleveland, Ohio), D-xylose (Sigma chemicals company, Poole, UK) D-cellobiose (Sigma chemicals company, Poole, UK) and D-glucose (BDH chemicals, Poole, UK) were prepared (100 g/l). Test carbohydrate solution (1 ml) was added in 9 ml sterilized basal media. Bacteria were grown on MRS agar plates at 37°C for 48 h, and a single colony was added in basal media containing different carbohydrates. Each tested bacteria were subcultured twice in identical medium. Growth of test cultures was monitored for 48 h by measuring absorbance at 600 nm using a spectrophotometer.

3.2.8 Encapsulation of *L. acidophilus*

Pea protein isolate (4.0% w/v; PPI) + alginate (0.5% w/v; AL) capsule was prepared by dissolving 40.0 g/L PPI in 0.1 M NaOH under constant mechanical stirring, and heating at 80ºC for 30 min before cooling to 40ºC in a water bath. Once cooled, the solution was then neutralized to pH 7.0 with 1.0 M HCl and 5.0 g/L AL powder was then dispersed into the solution. The biopolymer mixture was then heated at 80ºC for another 30 min, before being cooled to 40ºC in a water bath. The weights of the solutions were corrected for water loss during heating. A suspension containing 1.0 g washed *L. acidophilus* (~10^8 cfu/mL) was mixed with 18.0 g of the 4.0% PPI + 0.5% AL solution held at 40ºC and injected through a 20 G needle into 30 mL of filter sterilized 5.0% $CaCl_2$ + 1.0% (w/v) Tween 80 crosslinking bath. The resultant capsules were allowed to harden in the cross-linking solution for 30 min, and then washed three times with PS.

3.2.9 Survival of encapsulated *L. acidophilus* in SGJ

The survival of free and encapsulated *L. acidophilus* cells in SGJ (0.08 M HCl with 0.2 % (w/v) NaCl) were studied at pH 6.0 and 2.0, as described below. Aliquots of 0.5 mL of free and encapsulated *L. acidophilus* cells in PS were added to 9.5 mL of SGJ at 37ºC and incubated anaerobically. Samples containing SGJ at pH 6.0 were removed at 20 min intervals over the 2 h assay period; for samples containing SGJ at pH 2.0, tubes were removed at 5 min time intervals over the first 30 min, and thereafter at 30 min intervals until 2 h. Tubes containing SGJ at pH 2.0 were neutralized with 1.0 M NaOH upon removal from incubation. The contents of the tubes containing the encapsulated cells were homogenised (ES, Omni International Inc., Marietta, GA, USA) at a setting of 4.5 for 30s, serially diluted in PS and surviving bacteria enumerated by pour plating using MRS media. The plates were incubated under anaerobic conditions at 37ºC for 48 h. Experiments were conducted in triplicate.

3.2.10 Statistical analysis

Statistical analysis was carried out to see the differences between the experiments with a confidence interval of 95% by using analysis of variance (ANOVA). Comparison of means was carried out using Tukey's HSD test. All the experiments are repeated in triplicates (n=3).

3.3 Results

3.3.1 Probiotic resistance to SGJ

To determine the effect of prolonged exposure of probiotics to the acidic pH of the stomach, a 2 h *in vitro* SGJ survival assay was employed (Figure 3.3.1).

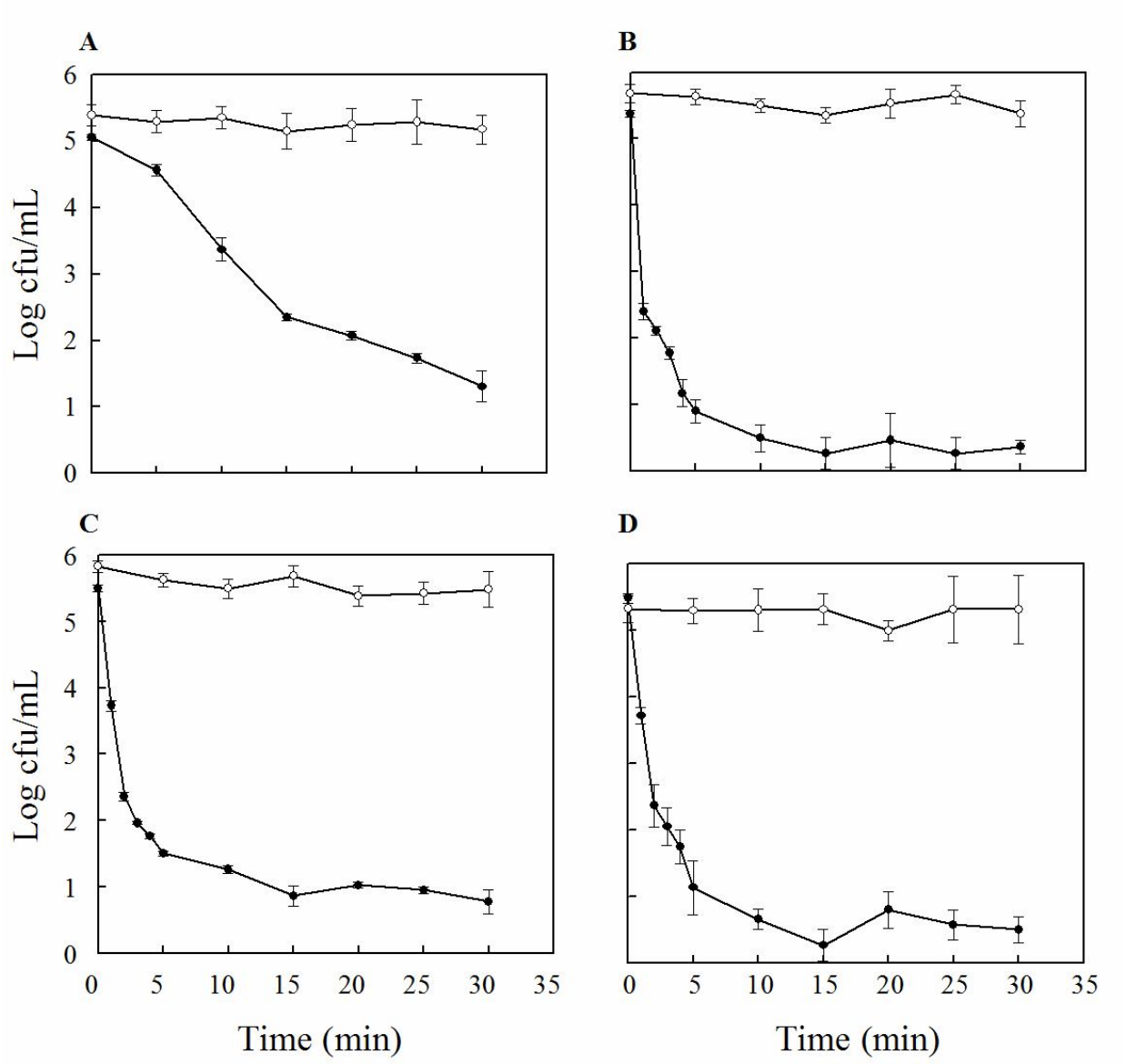

Figure 3.3.1 Survival of *L. acidophilus* (A), *B. adolescentis* (B), *B. catenulatum* (C), and *B. infantis* (D) in SGJ pH 2.0 (●) and pH 6.0 (○). The data are expressed as mean ± one standard deviation (n=3)

Results from these experiments demonstrated that *L. acidophilus* ATCC 11975 was the most resistant (*D*-value 10.2 ± 0.8 min) to SGJ (pH 2.0) (Table 3.3.1**),** but could not survive longer than 30 min of exposure under these conditions. The *D*-value of *L. acidophilus* was significantly ($P < 0.05$) more than other tested strains. Whereas, the viability of *L. acidophilus* was not significantly affected ($P > 0.05$) at pH 4.0 and 5.0 and remained viable even after 3 h of

exposure to SGJ (Figure 3.3.2). Plate count data revealed that none of the three *Bifidobacterium* spp. tested could survive more than 15 min of exposure to pH 2.0 SGJ, with viable cell numbers decreasing to undetectable levels in this period (Table 3.3.1).

Table 3.3.1 *D*-values (min) of free and encapsulated probiotics exposed to SGJ (pH 2.0). Data are expressed as the mean ± one standard deviation (n=3)

Bacteria	***D*-value (min)**
Survival studies	
*L. acidophilus**	10.17 ± 0.80^{a}
*B. catenulatum**	1.26 ± 0.02^{b}
*B. infantis**	1.15 ± 0.04^{b}
*B. adolescentis**	1.12 ± 0.03^{b}
Encapsulation studies	
L. acidophilus (Free) 0-30 min.	10.05 ± 0.19^{a}
L. acidophilus (Free) 30-120 min.	61.77 ± 4.25^{b}
L. acidophilus (Encapsulated) 0-30 min.	59.06 ± 4.91^{a}
L. acidophilus (Encapsulated) 30-120 min.	138.04 ± 8.04^{b}

[a,b] Means in columns within a category with unlike superscripts differ ($P < 0.05$). Statistical analysis was done separately for free and encapsulated cells.
*D-values were determined over the first 5 min due to the rapid depletion of viable free cells.

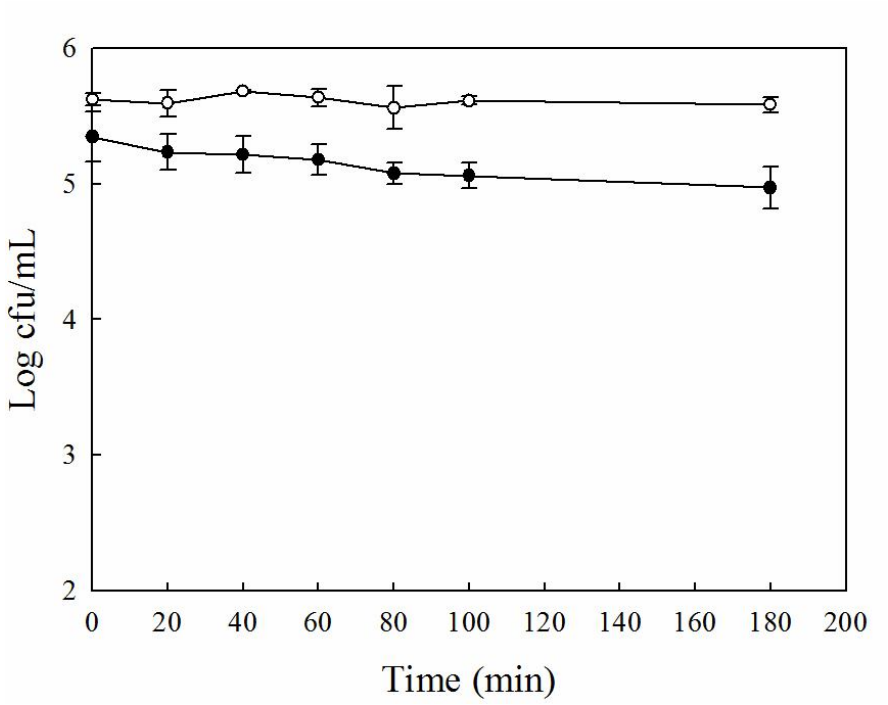

Figure 3.3.2 Survival of *L. acidophilus* in SGJ pH 4.0 (●) and pH 5.0 (○). The data are expressed as the mean ± one standard deviation (n=3)

3.3.2 Probiotic resistance to bile

The effect of Oxgall bile on the viability of the four probiotics is shown in figure 3.3.3; all test strains were able to survive for 5 h in MRS media supplemented with 0.3% bile. Optical density data showed that slow growth of all test strains occurred over a 24 h period in the presence of 0.3% bile-containing MRS (Figure 3.3.4). Following growth in bile-containing media, all tested probiotic strains produced unique crystalline structures, whereas no crystalline structures were observed when probiotics were added in control (Figure 3.3.5).

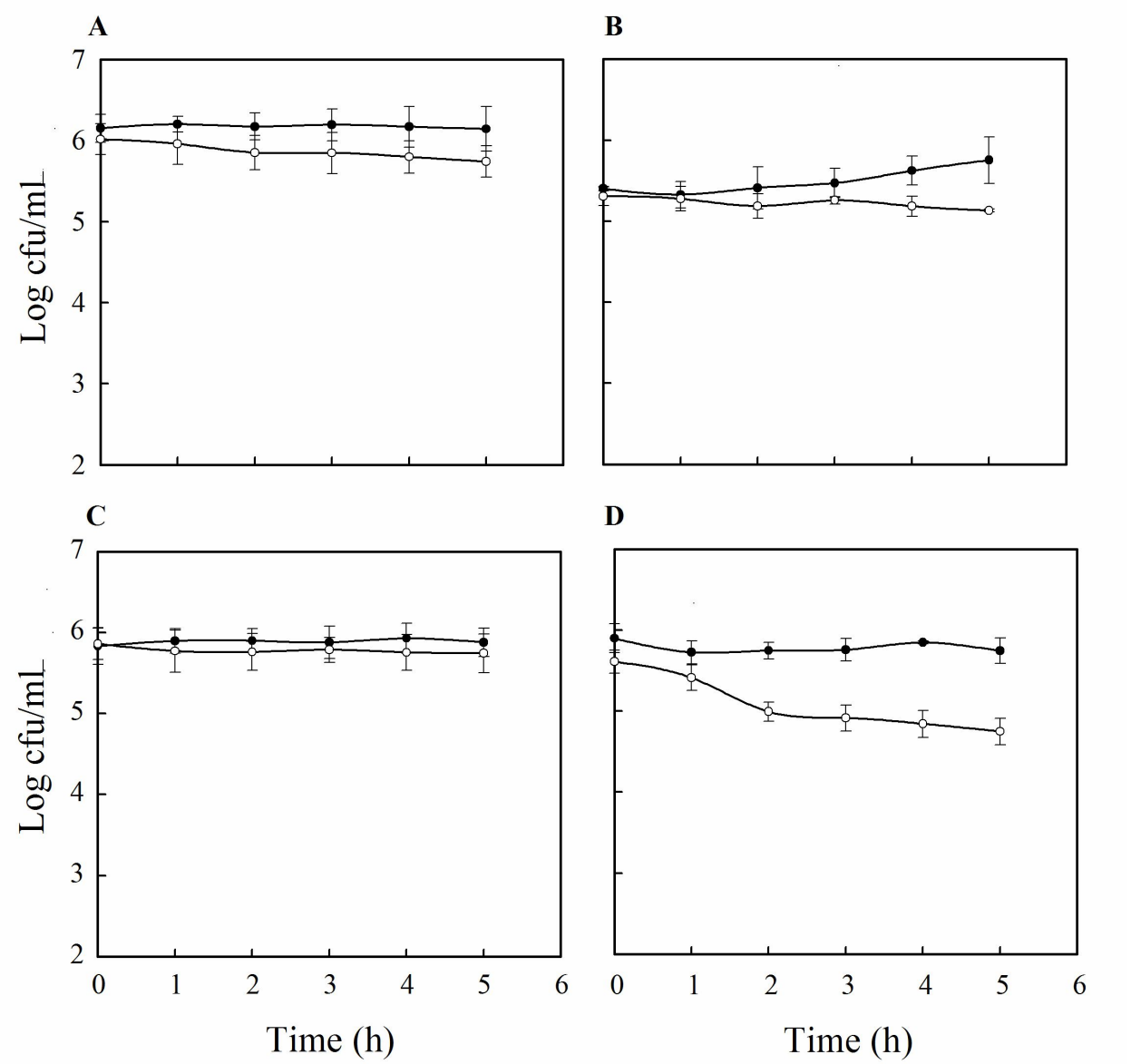

Figure 3.3.3 Survival of *L. acidophilus* (A), *B. adolescentis* (B), *B. catenulatum* (C), and *B. infantis* (D) in media containing 0.3% (w/v) Oxgall bile (○) and without added bile (●). The data are expressed as the mean ± one standard deviation (n=3)

Optical density results (Figure 3.3.4) suggest that slow growth of all test strains occurred in the presence of 0.3% bile containing MRS media, as compared to the control (MRS broth without added bile).

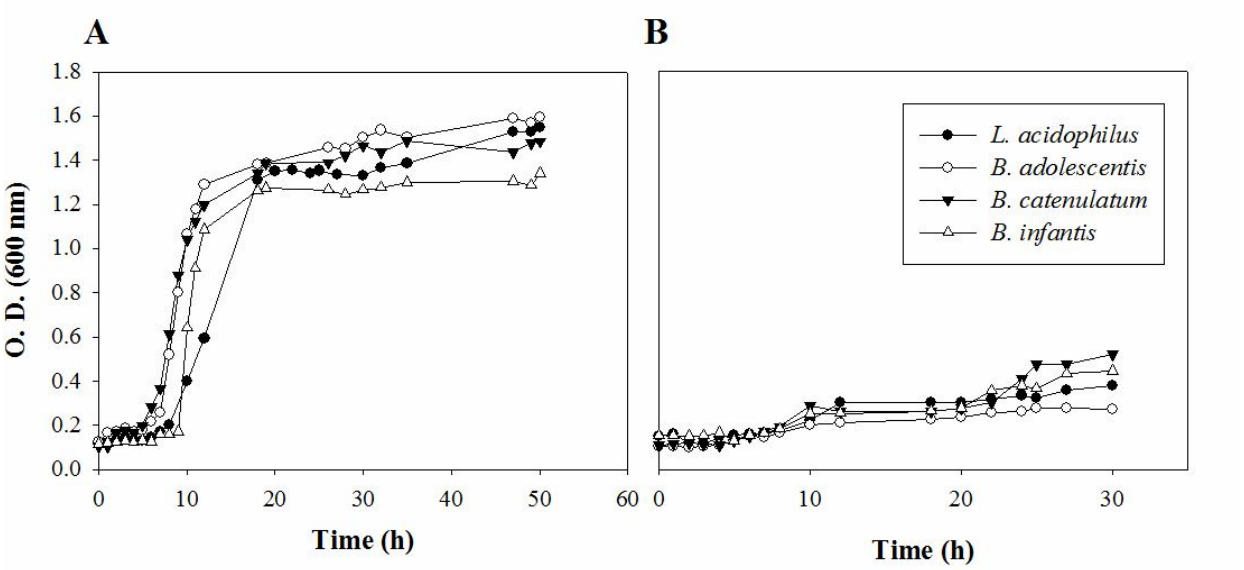

Figure 3.3.4 Growth of probiotics in A) MRS and B) 0.3% (w/v) Oxgall bile containing MRS media. The data are expressed as the mean ± one standard deviation (n=3)

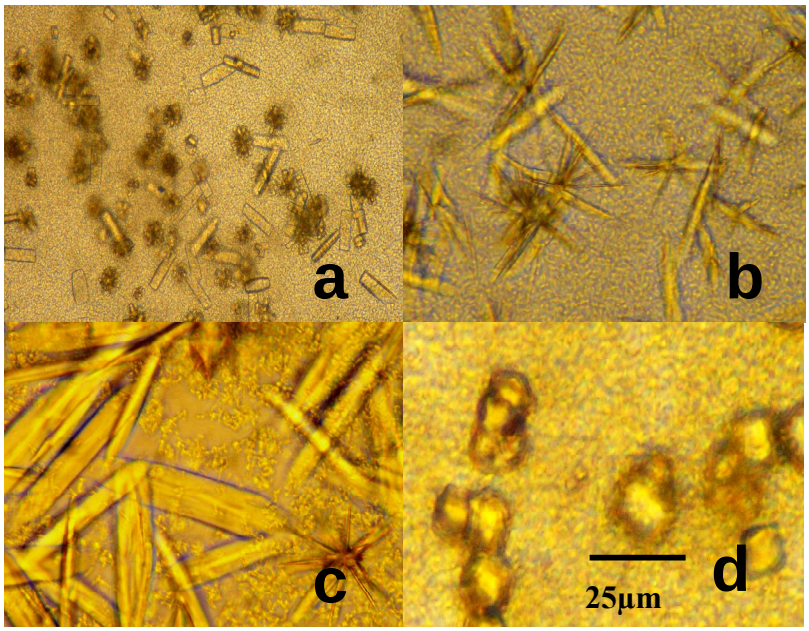

Figure 3.3.5 Light micrographs (magnification 400X NA 0.22) of crystals formed by test probiotic strains cultivated in MRS-broth media supplemented with 0.3% (w/v) Oxgall bile for 24 h incubation at 37ºC under anaerobic conditions. a) *L. acidophilus,* b) *B. adolescentis,* c) *B. catenulatum* and d) *B. infantis*

3.3.3 Probiotic adherence to Caco-2 cells

The *in vitro* adherence of probiotic bacteria to Caco-2 cells was determined, using a 2 h assay, to be species-specific (Figure 3.3.6). The initial number of bacterial cells added to each well was 5.4×10^5 cfu/mL. By the assay endpoint, *L. acidophilus* was shown to adhere to Caco-2 cells in the highest numbers (4.5×10^4 cfu/mL or ~10% of the added cells). In contrast, about 10-fold fewer *B. catenulatum* cells attached (3.1×10^3 cfu/mL), and *B. adolescentis* and *B. infantis* showed relatively poor adhesion with the number of adhering cells at the end of the assay being 2.6×10^1 and 1.5×10^1 cfu/mL, respectively. The viability of all tested probiotics was not effected in 5% CO_2-95% air atmosphere significantly ($P < 0.05$, data not shown).

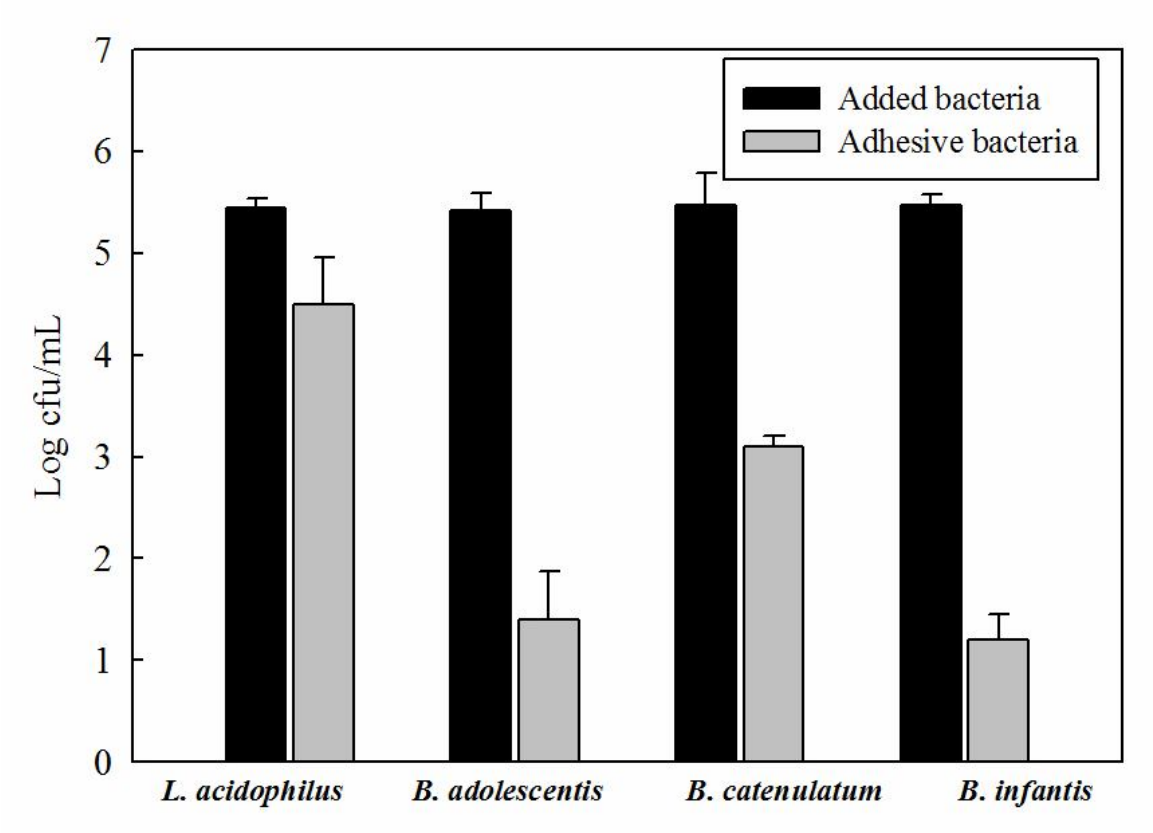

Figure 3.3.6 Adhesion of probiotic bacteria to Caco-2 cells. Data are expressed as the mean ± one standard deviation (n=3)

3.3.4 Probiotic susceptibility to antibiotics

It was determined that the patterns of antimicrobial susceptibility of the probiotic strains were quite variable (Table 3.3.2). *L. acidophilus* was found to be resistant to ciprofloxacin (4 μg/mL), nalidixic acid (32 μg/mL) and sulfisoxazole (256 μg/mL), and showed intermediate resistance to cefoxitin (4 μg/mL), amikacin (8 μg/mL), ceftriaxone (8 μg/mL), gentamicin (4 μg/mL), trimethoprim/sulphamethoxazole (1/19 μg/mL), and kanamycin (8 μg/mL). *B. catenulatum* was resistant to nalidixic acid (32 μg/mL) and kanamycin (64 μg/mL) and intermediately resistant to amikacin (4 μg/mL), ciprofloxacin (0.5 μg/mL), gentamicin (8 μg/mL), sulfisoxazole (64 μg/mL), trimethoprim/sulphamethoxazole (0.25/4.75 μg/mL), and streptomycin (32 μg/mL).

Table 3.3.2 Antibiotic resistance profiles of the tested probiotic strains

Antibiotic	**Conc. range, μg/mL**	***L. acidophilus***	***B. catenulatum***	***B. infantis***	***B. adolescentis***
Cefoxitin	32-0.5	I (4.0)	S	S	S
Amikacin	64-0.5	I (8.0)	I (4.0)	I (4.0)	S
Chloramphenicol	32-2	S	S	S	S
Tetracycline	32-4	S	S	S	S
Ceftriaxone	64-0.25	I (8.0)	S	I (0.25)	S
Amoxillin/Clavulanic-Acid	32/16-1/0.5	S	S	S	S
Ciprofloxacin	4-0.015	R	I (0.5)	I (0.25)	S
Gentamicin	16-0.25	I (4.0)	I (8.0)	I (2.0)	S
Nalidixic acid	32-0.5	R	R	R	I (0.5)
Ceftiofur	8-0.12	S	S	S	S
Sulfisoxazole	256-16	R	I (64.0)	I (16.0)	S
Trimethoprim/Sulphamethoxazole	4/76-0.12/2.38	I (1.0/19.0)	I (0.25/4.75)	I 0.25/4.75)	S
Kanamycin	64-8	I (8.0)	R	I (8.0)	R
Ampicillin	32-1	S	S	S	S
Streptomycin	64-32	S	I (32.0)	R	S

I, Intermediate (conc. μg/mL); S, Sensitive (No growth in any of the tested conc. range); R, Resistant (Growth in highest tested conc.)

B. infantis was resistant to streptomycin (64 μg/mL) and nalidixic acid (32 μg/mL) and showed intermediate resistance to amikacin (4 μg/mL), ceftriaxone (0.25 μg/mL), ciprofloxacin (0.25 μg/mL), gentamicin (2 μg/mL), trimethoprim/sulphamethoxazole (0.25/4.75 μg/mL), and kanamycin (8 μg/mL). Lastly, *B. adolescentis* was resistant to kanamycin (64 μg/mL) and showed intermediate resistance to nalidixic acid (0.5 μg/mL). None of the tested probiotics were resistant to chloramphenicol, tetracycline, amoxillin/clavulanic-acid, ceftiofur and ampicillin.

3.3.5 Carbon source utilization of probiotics

Growth of probiotics on different carbohydrates was tested by adding different carbohydrates in basal media (Figure 3.3.7). Carbon source utilization profiles of *L. acidophilus, B. catenulatum, B. infantis* and *B. adolescentis* are shown in Table 3.3.3, respectively.

All probiotics were able to utilize glucose (control) and lactose as sole carbon sources. Xylose was utilized by *B. adolescentis* and *B. catenulatum.* Cellobiose was utilized by *B. catenulatum* and *L. acidophilus,* where poor growth was observed by the later strain. Slow growth of *B. adolescentis* and *B. infantis* was observed with methyl-D-glucoside, with a longer lag phase (~ 20 h). Analysis of Ecoplate results indicated that all strains grew to the highest culture optical densities on D-xylose. *B. catenulatum* utilized a number of carbohydrates including β-methyl-D-glucoside, D-cellobiose, and α-D-lactose.

Table 3.3.3 Optical densities at 590 nm attained after 48 h by probiotics during growth on different sole carbon sources. The data are expressed as the mean ± one standard deviation (n=3)

Carbon name	*B. infantis*	*L. acidophilus*	*B. adolescentis*	*B. catenulatum*
2-hydroxybenzoic acid	0.38 ± 0.03^{a}	0.34 ± 0.03^{ab}	0.29 ± 0.00^{b}	0.38 ± 0.00^{b}
4-hydroxybenzoic acid	0.38 ± 0.02^{a}	0.34 ± 0.02^{ab}	0.27 ± 0.04^{b}	0.39 ± 0.03^{b}
D, L-α-glycerol phosphate	0.28 ± 0.01^{a}	0.30 ± 0.09^{a}	0.28 ± 0.00^{a}	0.37 ± 0.02^{a}
D-cellobiose	0.32 ± 0.04^{a}	0.37 ± 0.07^{a}	0.35 ± 0.02^{a}	0.69 ± 0.04^{b}
D-galactonic acid γ-lactone	0.31 ± 0.04^{a}	0.38 ± 0.00^{b}	0.27 ± 0.03^{a}	0.37 ± 0.02^{b}
D-galacturonic acid	0.34 ± 0.04^{a}	0.40 ± 0.03^{ab}	0.31 ± 0.01^{b}	0.40 ± 0.02^{b}
D-glucosaminic acid	0.30 ± 0.02^{a}	0.37 ± 0.02^{ab}	0.28 ± 0.07^{ab}	0.40 ± 0.01^{b}
D-malic acid	0.38 ± 0.14^{a}	0.37 ± 0.02^{a}	0.33 ± 0.04^{a}	0.37 ± 0.01^{a}
D-mannitol	0.38 ± 0.04^{a}	0.36 ± 0.05^{a}	0.32 ± 0.01^{a}	0.40 ± 0.02^{a}
D-xylose	1.28 ± 0.16^{a}	0.92 ± 0.02^{a}	0.98 ± 0.10^{ab}	1.21 ± 0.12^{b}
Glucose-1-phosphate	0.37 ± 0.06^{a}	0.34 ± 0.03^{a}	0.31 ± 0.02^{a}	0.39 ± 0.02^{a}
Glycogen	0.32 ± 0.02^{a}	0.34 ± 0.02^{ab}	0.31 ± 0.02^{ab}	0.37 ± 0.03^{b}
Glycyl-L-glutamic acid	0.33 ± 0.00^{a}	0.37 ± 0.02^{b}	0.30 ± 0.01^{c}	0.38 ± 0.01^{c}
i-erythritol	0.38 ± 0.03^{a}	0.40 ± 0.09^{a}	0.28 ± 0.04^{a}	0.39 ± 0.01^{a}
Itaconic acid	0.35 ± 0.03^{a}	0.38 ± 0.04^{ab}	0.30 ± 0.04^{ab}	0.39 ± 0.00^{b}
L-arginine	0.32 ± 0.07^{a}	0.39 ± 0.02^{a}	0.28 ± 0.02^{a}	0.35 ± 0.03^{a}
L-asparagine	0.32 ± 0.03^{a}	0.36 ± 0.04^{ab}	0.29 ± 0.01^{bc}	0.40 ± 0.02^{c}
L-phenylalanine	0.46 ± 0.04^{a}	0.37 ± 0.05^{ab}	0.34 ± 0.01^{bc}	0.47 ± 0.04^{c}
L-serine	0.40 ± 0.01^{a}	0.38 ± 0.06^{ab}	0.29 ± 0.05^{ab}	0.39 ± 0.00^{b}
L-threonine	0.36 ± 0.03^{a}	0.38 ± 0.05^{a}	0.33 ± 0.05^{a}	0.41 ± 0.01^{a}
N-acetyl-d-glucosamine	0.38 ± 0.03^{a}	0.52 ± 0.06^{a}	0.30 ± 0.02^{ab}	0.41 ± 0.05^{b}
Phenylethlamine	0.35 ± 0.01^{a}	0.34 ± 0.01^{b}	0.29 ± 0.01^{bc}	0.37 ± 0.01^{c}
Putrescine	0.35 ± 0.08^{a}	0.34 ± 0.02^{a}	0.29 ± 0.02^{a}	0.31 ± 0.10^{a}
Pyruvic acid methyl ester	0.30 ± 0.01^{a}	0.47 ± 0.03^{ab}	0.22 ± 0.09^{bc}	0.36 ± 0.01^{c}
Tween 40	0.32 ± 0.06^{a}	0.27 ± 0.09^{a}	0.28 ± 0.06^{a}	0.37 ± 0.17^{a}
Tween 80	0.27 ± 0.07^{a}	0.29 ± 0.10^{a}	0.28 ± 0.05^{a}	0.45 ± 0.09^{a}
water	0.30 ± 0.03^{a}	0.35 ± 0.03^{a}	0.27 ± 0.03^{a}	0.32 ± 0.09^{a}
α-cyclodextrin	0.32 ± 0.04^{a}	0.34 ± 0.02^{ab}	0.29 ± 0.01^{ab}	0.38 ± 0.05^{b}
α-D-lactose	0.37 ± 0.13^{a}	0.40 ± 0.07^{a}	0.44 ± 0.01^{a}	0.97 ± 0.07^{b}
α-Ketobutyric acid	0.36 ± 0.01^{a}	0.39 ± 0.05^{a}	0.29 ± 0.05^{a}	0.37 ± 0.03^{a}
β-methyl-D-glucoside	0.33 ± 0.04^{a}	0.47 ± 0.03^{b}	0.37 ± 0.08^{ab}	0.82 ± 0.01^{c}
γ-hydroxybutyric acid	0.44 ± 0.02^{a}	0.42 ± 0.08^{a}	0.35 ± 0.00^{a}	0.44 ± 0.03^{a}

^{a,b,c} Means in a row within a category with unlike superscripts differ ($P < 0.05$).

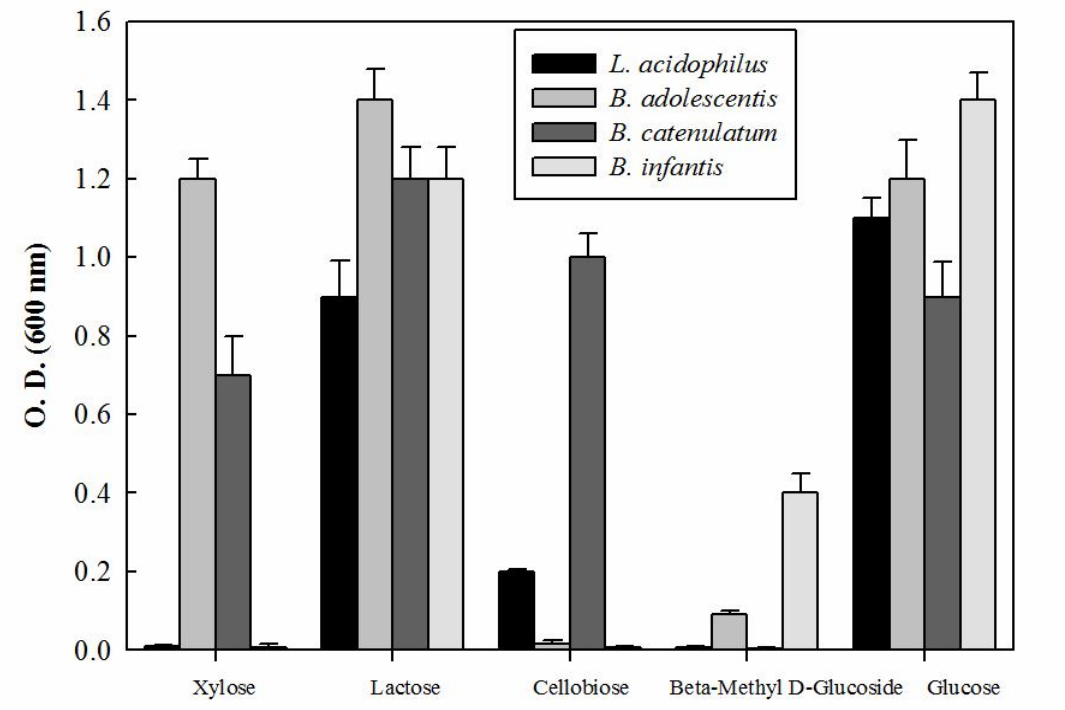

Figure 3.3.7 Maximum optical densities (O.D. 600) attained by probiotics during growth on different carbon sources. Data are expressed as the mean ± one standard deviation (n=2)

3.3.6 Survival of encapsulated *L. acidophilus* in SGJ

L. acidophilus was selected for encapsulation and subsequent challenge, *in vitro,* to simulated gastric conditions (pH 2.0). The PPI-AL microcapsules, prepared by extrusion, were teardrop in shape and uniform in size (~3 mm) (Figure 3.3.8). The survival of *L. acidophilus* in acidic condition is shown in Figure 3.3.9, and clearly demonstrates a protective effect of encapsulation during the 2 h exposure to simulated gastric conditions with only a ~1 log cfu/mL loss in cell viability. Whereas, unprotected *L. acidophilus* cells underwent a >6 log cfu/mL loss in cell viability over the same period ($P < 0.05$). Comparing *D*-values of the encapsulated and un-encapsulated cells (Table 3.3.1) revealed that the PPI-AL matrix improved survival by almost 6 times (59.0 ± 5.0 min versus 10.1 ± 0.2 min; *D*-value for the free cells calculated over the first 30 min).

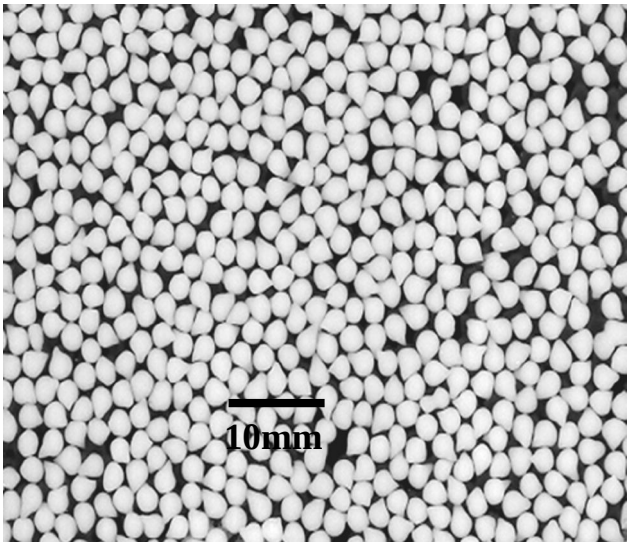

Figure 3.3.8 Light micrograph of pea protein-alginate beads prepared by extrusion

Viability of *L. acidophilus* cells remained relatively unchanged for both free and encapsulated cells for a period of 2 h incubation at pH 6.0.

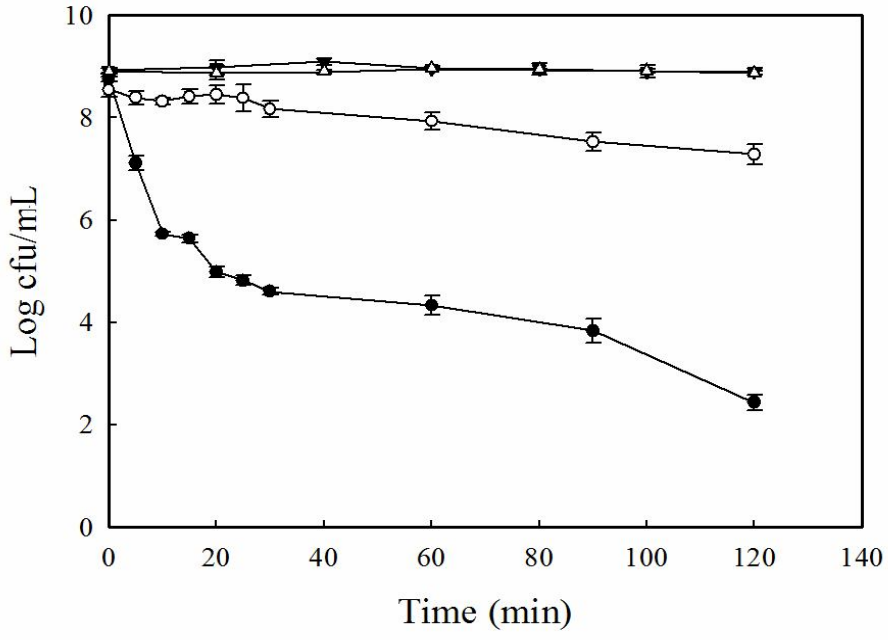

Figure 3.3.9 Survival of free (solid symbols) and encapsulated (open symbols) *L. acidophilus* in SGJ of pH 2.0 (●, ○) and pH 6.0 (▼, Δ). The data are expressed as the mean ± one standard deviation (n=3)

3.4 Discussion

The human GIT is a complex microbial community consisting of ~10^{10}-10^{14} cfu of bacteria per gram of faecal material. Given such high numbers and diversity, it is not surprising that the composition and numbers of these organisms should play a significant role in human health and disease. Within this community, probiotics (lactobacilli and bifidobacteria) have been studied for their health promoting and otherwise beneficial effects (Dunne et al., 2001; Goktepe et al., 2006). However, for probiotics to exert beneficial effects in the GI tract, they must survive transit through the stomach. Each day, the human stomach secretes about 3 L of gastric juice with a pH of around 2.0. The transit time of food in the stomach is between 2 and 4 h (Smith, 1995); liquids take about 20 min to pass through the stomach (GastroNet Australia, 2001). Hence, it is necessary for probiotic microorganisms, if unaided or unprotected, to tolerate the acidic conditions of the stomach for periods of up to 4 hrs so that they may reach the colon in a viable state. Lactobacilli of intestinal origin are considered intrinsically-resistant to acidic environments and are often employed in fermented foods as probiotics. In order to improve the numbers of surviving probiotics during gastric transit, components such as milk (Conway et al., 1987), milk proteins (Charteris et al., 1998a), cheese and yogurt (Gardiner et al., 1999), reconstituted skim milk with gum acacia (Desmond et al., 2002), and cereal extracts have been used (Charalampopoulos et al., 2003). However, actual *in vivo* data demonstrating this enhanced survival is lacking. Figure 3.3.1 shows that simulated gastric conditions with a pH of 2.0 were lethal in as little as 10 min for all unprotected probiotics tested, with the exception of *L. acidophilus* which survived for 30 min. Thus, *L. acidophilus* was most likely to survive passage through the stomach compared with the other probiotics tested in this study. For *Bifidobacteria* spp., transit through the stomach gastric conditions would only be possible if the pH was raised to above 4.0 if, for example, the organism was ingested along with a meal.

Raising the pH to 4.0 or 5.0 also significantly improved the survival kinetics of *L. acidophilus* strains (Figure 3.3.2); this would likely be a viable strategy for improving survival of probiotics through the stomach environment.

Bile tolerance by probiotics has been shown to be strain- and bile type-dependent, with resistance levels ranging from bile concentrations of 0.125 to 2.0% (Lian et al., 2003; Margolles et al., 2003). While all strains involved in this study could tolerant 0.3% Oxgall bile (Figure 3.3.3), there were some minor differences. In general, the required concentration of bile salts considered necessary to screen for resistant strains for human use is 0.3%.

In the current study, crystalline floccules were found in broth media containing bile (Figure 3.3.4) and were species specific. Zavaglia et al. (2002) suggested that when cultures are exposed to bile, changes take place in the cell membrane causing auto-aggregation and subsequently, flocculation. Metabolic actions of the live cells or the work of enzymes released from lysed dead cells in the culture medium could induce a chemical reaction which makes the bile component of the medium insoluble. The significance of the different shapes of crystals formed by the tested probiotic panel in current study is not yet clear and to my knowledge has not previously been described.

The adhesion of probiotic strains to intestinal epithelial cells is considered as a prerequisite feature for attachment and proliferation in the intestinal environment (Kailasapathy and Chin, 2000). Adhesion has also been cited as important for transient colonization (Alander et al., 1999), enhanced healing of the damaged gastric mucosa (Elliott et al., 1998), modulation of the immune system (Perdigon et al., 2002) and antagonism against pathogens (Jin et al., 2000). Caco-2 cell lines have seen extensive use as an *in vitro* model of the human intestinal epithelium for screening probiotic adherence (Gopal et al., 2001), and culture plating has been employed to

count the total adhering bacteria to Caco-2 cells (Forestier et al., 2001; Matijasic et al., 2003; Bertazzoni-Minelli et al., 2004; Pennacchia et al., 2006). Previously, the adhesion of *L. casei rhamnosus* Lcr35 was studied using an initial bacterial inoculum of 10^9 cfu/well, where the final number of viable adhering bacterial cells was a little less than 10^5 cfu/well (Forestier et al., 2001). In a second study, adhesion percentages of six *L. casei* strains were calculated, in which four had an adhesion percentage ranging from 0.3 to 0.74 (Bertazzoni-Minelli et al., 2004). Two *L. gasseri* strains of human origin, K7 and LF221 showed good adhesion with Caco-2 cells, when added with an initial concentration of 10^9 cfu/well, yielding final numbers of viable adhering bacterial cells of 10^7 cfu/well (Matijasic et al., 2003). Finally, the eight *Lactobacillus* strains belonging to the *L. plantarum*-group were analysed by Pennacchia et al. (2006). The number of final adhering bacteria was ~10^7 cfu/mL when 10^9 cfu/well was added initially. In the present study, the probiotic strains examined were shown to attach to the Caco-2 cells *in vitro* with varying success, as shown in figure 3.3.6. Clearly, *L. acidophilus* showed higher or similar adhesion levels to those results described above (Forestier et al., 2001; Matijasic et al., 2003; Bertazzoni-Minelli et al., 2004; Pennacchia et al., 2006). Among the *Bifidobacterium* strains tested *B. catenulatum* attached considerably more strongly to Caco-2 cells in comparison with *B. adolescentis* and *B. infantis*, suggesting the latter strains may have limited use as probiotics when viewed from the adhesion perspective.

It has also been reported that some lactobacillus strains can strongly adhere to the gut mucosa, where they subsequently interfere with the adhesion of pathogenic bacteria to intestinal cells (Servin, 2004). Adherence to the intestinal epithelial cells is also an important requirement for colonization by micro-organisms, and for bacterial pathogens, which is a pivotal step for virulence (Westerlund and Korhonen, 1993). The ability of some lactobacillus strains to

co-aggregate with intestinal pathogens in intestinal lumen might prevent pathogens from reaching the intestinal mucosa. At the same time, production of antimicrobial compounds by probiotics helps to kill enteric pathogens and ultimately reduce their population in the GIT. As adhesion to intestinal tissue has been considered to be one of the cornerstones of probiotic function, it was considered in the present work as one of the main criteria for probiotic selection for encapsulation; thus, *L. acidophilus*, which attached at a rate of ~10% of the cells used in the assay, was the most successful probiotic of the tested panel. However, there is a lack data indicating that probiotic gut colonization occurs, or that it is even necessary for a probiotic effect to be realized (Tappenden and Deutsch, 2007).

There is limited information on the resistance of probiotic bacteria to antimicrobials of clinical importance and even a more limited understanding of how these resistance factors influence probiotic reproductive success. Antibiotic resistance of probiotics might be a desirable feature, as it could potentially aid in their survival in the GIT, especially when used after antibiotic therapy or in co-administration with antibiotics to restore intestinal health (Salminen et al., 1998a). Alternatively, resistance to these agents could increase the spread of resistance within the dense, diverse anaerobic microbial population (including pathogens) inhabiting the GIT of humans and animals (Scott, 2002). The antibiotic susceptibility system used in this study is a microtiter plate-based version of the classic broth dilution method for testing for antibiotic susceptibility, and provided both qualitative and quantitative susceptibility results. All tested probiotics were resistant to Gram-negative spectrum-antibiotic nalidixic acid (conc. 32 μg/mL), whereas, *B. adolescentis* showed intermediate resistance (Table 3.3.2). Charteris et al. (1998b) reported resistance of *B. adolescentis* and *B. infantis* to nalidixic acid (30 μg/mL). Kanamycin resistance (30 μg/disk) and sensitivity to the broad-spectrum antibiotics chloramphenicol

(32 μg/disk) and tetracycline (30 μg/disk) of tested *Lactobacilli* and *Bifidobacteria* strains were reported by Zhou et al. (2005). Similarly, all probiotics tested in the present study were sensitive to chloramphenicol (32 μg/mL) and tetracycline (32 μg/mL), *B. catenulatum and B. adolescentis* were resistant to kanamycin conc. 64 μg/mL, whereas, *L. acidophilus* and *B. infantis* showed intermediate resistance to kanamycin. *L. acidophilus*, *B. catenulatum* and *B. infantis* showed intermediate resistance to the combination of trimethoprim/sulphamethoxazole. Studies by Masco et al. (2006) showed reduced resistance of bifidobacteria against a therapeutic combination of trimethoprim and sulphamethoxazole due to their synergistic inhibitory effect on thymidine synthesis.

At any given time, the human colon and particularly the proximal bowel contain many different types of carbohydrates. Consequently, bacteria which can adapt rapidly to the changing substrate availability and grow on a variety of carbon sources will have a competitive advantage in the GIT ecosystem (Macfarlane et al., 2008). Carbon substrate utilization was highly variable among tested probiotics and considerable inter-species and inter-strain differences existed, as shown in Table 3.3.3. While individual probiotic species exhibited unique carbohydrate substrate preferences, several trends in carbon source utilization emerged from this study. For example, the highest culture optical densities were recorded by *B. catenulatum* (Table 3.3.3) for a number of carbohydrates, including β-methyl-D-glucoside, D-cellobiose, D-xylose and α-D-lactose. Similarly, rapid growth of *B. catenulatum* was observed in the presence of lactose and xylose (Hopkins et al. 1998). Fermentation of cellobiose by *B. catenulatum* strains was previously reported by Salminen et al. (1998a); whereas, *B. adolescentis* and *B. infantis* did not ferment cellobiose (Roy and Ward, 1990). Low utilization of Tween-40 and Tween-80 were recorded for *L. acidophilus* and *B. infantis*. Similarly, maximum specific growth rates of 0.25 and 0.29 were

recorded by *B. catenulatum* and *B. adolescentis* strains with xylose; whereas, *B. infantis* did not show appreciable growth in the presence of xylose (Hopkins et al., 1998). In the present study, the highest optical densities for all the probiotics tested were observed when D-xylose was provided as the sole carbons source. The differences in carbon source utilization patterns observed in this study may have particular interest from the viewpoint of future prebiotic development. Use of such compounds to target specific organisms in the large intestine would be of great value for defined health-promoting purposes in future.

Buffering capacities, carbohydrate and protein constituents, and encapsulation are some of the technologies that have been used previously for increasing probiotic survival in acidic environments (Corcoran et al., 2005). Encapsulation is currently seeing intense focus as a physical means for protecting probiotics from the harsh effects of the gastric environment (Mortazavian et al., 2007). Microencapsulation using cellulose acetate phthalate protected bifidobacteria quite notably during spray drying and during prolonged exposure to simulated gastric conditions (Favaro-Trindale and Grosso, 2002). Emulsification, gelation and film formation are some of the properties which supports the technological utilisation of pea proteins (Choi and Han 2002; Rangel et al. 2003). Pea protein has also been utilised for microencapsulation of α-tocopherol (Pierucci et al., 2007) but to my knowledge has not been used for entrapping live microorganisms. Transformation towards strictly plant-based protein materials would be advantageous, as probiotics ingredients could be then incorporated into non-dairy products, or have greater acceptance in materials that restrict animal-derived materials (eg; gelatin, whey). While a number of capsule matrices have been explored, the cross-linked alginate matrix system has most frequently seen application for LAB encapsulation (Sheu et al., 1993). However, these systems appear to be subject to a more rapid degradation and release of

active ingredients at low pH (Gombotz and Wee, 1998). Lee and Heo (2000) showed that *Bifidobacterium longum* encapsulated in calcium alginate containing 2.0, 3.0, and 4.0% sodium alginate survived exposure to SGJ (pH 1.5) significantly better than free cells. The death rate of the cells in the beads decreased proportionally with an increase in both the alginate gel concentration and bead size. However, survival of probiotics in alginate starch microcapsules was improved during refrigerated storage in yoghurt but not after exposure to acid and bile solutions (Sultana et al., 2000). Microencapsulated *L. acidophilus* strains showed 2-3 log improvement in viability after 3h of incubation at pH 2.0, when compared to free cells (Chandramouli et al. 2004). Similarly, non-encapsulated cells were completely destroyed when exposed to artificial gastric juice of pH 1.2 and 1.5 for 3 h, while the treatment reduced the viable count of encapsulated cells only by 3 log units (Kim et al. 2008). In the present study (Figure 3.3.9), encapsulation of *L. acidophilus* within pea protein – alginate capsules resulted in an approximate 6-fold increase in the survival of bacteria in pH 2.0 SGJ.

3.5 Connection to the next study

Preliminary screening of co-aggregation potential may be useful to identify potential probiotic strains suitable for food, human or animal use. Co-aggregation of probiotics with pathogens could potentially interfere with pathogen colonization of the GIT, survival (through various antimicrobial mechanisms) and subsequent manifestation of illness. In addition, co-aggregation of probiotics with other established commensal GIT microflora may aid in the attachment and colonization of probiotics to mucosal surfaces. Thus, the next study was performed to elucidate the aggregation and antibacterial properties of tested probiotic strains.

4 In vitro growth control of *Escherichia coli* O157:H7 and *Clostridium sordelli* ATCC 9714 by the probiotic *Lactobacillus acidophilus* ATCC 11975

Abstract

A panel of probiotic strains, including *Lactobacillus acidophilus* ATCC 11975, *Bifidobacterium infantis* ATCC 15697D, *Bifidobacterium catenulatum* ATCC 27675 and *Bifidobacterium adolescentis* ATCC 15703, were screened, using the agar spot test for their *in vitro* antibacterial activity against *Escherichia coli* O157:H7 and *Clostridium sordelli* ATCC 9714. Based on preliminary findings, *L. acidophilus* was then examined in more detail for its *in vitro* co-aggregation and antimicrobial activity against *E. coli* O157:H7 and *Cl. sordelli*. Cell surface properties of probiotic and pathogen strains were characterized in terms of adhesion to hydrocarbons (BATH), and their relative auto- and co-aggregation abilities. *Cl. sordelli* showed the highest 66.0 ± 5.5 % hydrophobicity and was also the most successful auto-aggregating strain, showing 63.6 ± 1.4 % auto-aggregation after 24 h incubation at 37°C under anaerobic conditions. *L. acidophilus* was able to co-aggregate with both tested pathogens and showed 47.9 ± 3.6 % and 51.8 ± 4.2 % co-aggregation with *E. coli* and *Cl. sordelli*, respectively. Bactericidal activity of probiotic supernatant (cell-free) was observed, as the number of viable bacteria was reduced over 24 h by approximately 4 log and 2 log cfu/mL for *E. coli* and *Cl. sordelli*, respectively. In general, *L. acidophilus*-mediated inhibition of *E. coli* was greater than that of *Cl. sordelli.* Antagonistic activity of the probiotic strain appeared to be partially pH-independent, as the use of buffered MRS (containing 0.2% sodium bicarbonate) agar media did not completely eliminate the inhibitory activity. Antagonistic activity was shown to be related to heat-stable non-proteinaceous compound(s), which were resistant to heating at 100°C for 10 min and to trypsin (1 mg/mL).

4.1 Introduction

The use of LAB as probiotics for the therapeutic treatment of a broad number of gastrointestinal conditions and infectious diseases (e.g., rotavirus diarrhea, influenza virus and *Helicobacter pylori*) has increased over the last few years (Goktepe et al., 2006). Lactobacilli and bifidobacteria, the most commonly-cited probiotic genera, reportedly play a significant role in maintaining the intestinal microflora and stimulating the immune system of its host (Saarela et al., 2002). When present in sufficient numbers (10^7 cfu/g or mL), probiotics are believed to create a healthy equilibrium between beneficial and potentially harmful microflora in the gut (Suskovic et al., 2001). *Lactobacillus* spp., such as *L. acidophilus, L. bulgaricus*, *L. lactis* along with *Bifidobacterium bifidum* and *Streptococcus faecium* constitute an integral part of the healthy gastro-intestinal microecology (Fernandes et al., 1987).

Antimicrobial substances produced by probiotics, either singly or in combination, may have bactericidal or bacteriostatic effects on other microbial populations. These antimicrobial effects can alter the proliferation of undesired pathogens by influencing the outcome of competition for chemicals or available energy (Fredrickson and Stephanopoulos, 1981). Production of antibacterial substances such as antibiotics (Shahani et al., 1976), bacteriocins (Barefoot and Klaenhammer, 1983; Millette et al., 2007), diacetyl and ammonia (Vandenbergh, 1993), hydrogen peroxide (Dahiya and Speck, 1967; Reid, 2000) and organic acids such as acetic, propionic and lactic acid (Naaber et al., 2004) by certain *L. acidophilus* strains have been reported. The *in vitro* production of inhibitory compounds toward known pathogens by various probiotic species has often been used in the selection of candidate probiotic strains.

In vitro inhibitory activity against a wide range of bacteria, including *Clostridium* spp., *Bacteroides* spp., *Bifidobacterium* spp., *Enterobacteriaceae* spp., *Pseudomonas* spp. *Staphylococcus* spp., and *Streptococcus* spp., has been shown by *Lactobacillus* spp. strain GG,

isolated from the human GI tract (Silva et al., 1987). *Lactobacillus gasseri*, which is considered to be a dominant inhabitant of the human intestine, was found to produce a bacteriocin that exhibited a wide spectrum of bactericidal activity against enteric pathogens. Early studies explored the use of oral preparations of viable *Lactobacillus acidophilus* in the treatment of functional gastrointestinal disturbances (Rafsky and Rafsky, 1955). Subsequently, Vincent et al. (1959) found that *L. acidophilus* produced lactocidin, a substance with broad antibacterial properties.

Co-aggregation is a process by which genetically-distinct bacteria become attached to one another via specific lectin-like adhesins and receptor molecules. Only a limited number of studies have examined probiotic-pathogen co-aggregation, on the basis that it might be an important protective mechanism against pathogen colonization in the gastrointestinal tract (GIT) (Reid et al., 1988; Schellenberg et al., 2006; Collado et al., 2008). Cumulative evidence, especially from oral microbiology work, suggests that aggregation influences the development of complex multispecies biofilms (Rickard et al., 2003). Bacterial aggregation between microorganisms of the same strain (auto-aggregation) or between genetically-divergent strains (co-aggregation) is likely of considerable importance in the human GIT, where mechanisms for colonizing the epithelial lining, and the organisms that inhabit it, would likely be necessary for any subsequent probiotic effect (Collado et al., 2008). A relationship between auto-aggregation and adhesion ability has been reported for some bifidobacterial spp., and a correlation between adhesion ability and hydrophobicity has been observed in some lactobacilli (Del Re et al., 2000). Furthermore, it has been suggested (Collado et al., 2007) that co-aggregation of bacteriocin-producing LAB with pathogens, may constitute an important host-defence mechanism against

infection. Co-aggregation with potential gut pathogens could therefore contribute to the probiotic properties ascribed to specific LAB.

The objective of the present study was to determine cell surface hydrophobicity, auto-aggregation and pathogen co-aggregation properties of probiotic strain *L. acidophilus* ATCC 11975. Antibacterial properties of this probiotic strain were then evaluated against selected pathogens.

4.2 Materials and methods

4.2.1 Bacteria and culture conditions

The probiotic strains *Lactobacillus acidophilus* ATCC 11975, *Bifidobacterium infantis* ATCC 15697D, *Bifidobacterium catenulatum* ATCC 27675 and *Bifidobacterium adolescentis* ATCC 15703 were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and stored at -80°C using 10% glycerol amended in culture broth as a cryoprotectant. Lactobacilli De Man Rogosa and Sharpe (MRS) broth (VWR International Ltd., Mississauga, ON) was used as a culture media for lactobacilli; whereas, MRS supplemented with 0.05% w/v L-cysteine HCl was used for bifidobacteria. Prior to each assay, stock cultures were propagated twice in fresh MRS broth and then incubated anaerobically (10% H_2, 10% CO_2, and 80% N_2) at 37°C for 24 h. Seed culture was harvested at the end of the exponential phase of growth (approximately 20 h) by centrifugation at 3,250 × g (Model 5810 R equipped with a swing-bucket rotor A-4-81; Eppendorf, Hamburg, Germany) for 10 min at 4°C and washed twice with phosphate-buffered saline (PBS, 4.3 mM $Na_2HPO_4.7H_20$, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.2) and resuspended in peptone saline (PS, 8.5 g/L NaCl, 1.0 g/L peptone, pH 7.0). *Escherichia coli* O157:H7 strain B1 was grown in Luria-Bertani medium (LB, Sigma-Aldrich Canada Ltd., Oakville, ON) and incubated aerobically at 37°C. *Clostridium sordelli* ATCC 9714

was grown in Reinforced Clostridial Medium (RCM, Oxoid Ltd., Hampshire, UK) and incubated anaerobically. All anaerobic culture work was conducted using an anaerobic chamber (Forma Scientific, Inc., Model 1025, Marietta, OH) maintained at 37°C.

4.2.2 Detection of antimicrobial activity

This assay was a modification of the agar spot test described by Jacobsen et al. (1999). Test probiotic cultures were spotted (~ 2 µL) on the surface of MRS agar and on MRS agar containing 2 g/L $NaHO_3$ (Fisher Scientific Co., Fair Lawn, NJ) to neutralize any acid effect (Toure et al., 2003). Plates were incubated anaerobically at 37°C for 24 h to develop the colony spots. Anaerobic conditions were used to minimize the formation of hydrogen peroxide and acetic acid. The inhibitory effect of MRS was tested as a negative control on each plate. A 100 µL volume of an overnight culture of the indicator (pathogen) bacteria was then mixed with 7 mL of soft agar (0.7% agar), using Luria-Bertani (LB) agar for *E. coli* O157:H7, and RCM for *Clostridia* spp., and poured over the probiotic spot. The plates were incubated anaerobically (for *Clostridia* spp.) or aerobically (for *E. coli* O157:H7) at 37ºC for an additional 24 h, after which inhibition zones were quantified in centimeters. Each test was performed in triplicate.

4.2.3 BATH (Bacterial adhesion to hydrocarbons) test

The bacterial adhesion to hydrocarbons (BATH) test was performed according to the method of Collado et al. (2008) with minor modifications. Overnight cultures were harvested by centrifugation at 3,250 × g (Model 5810 R with swing-bucket rotor A-4-81; Eppendorf, Hamburg, Germany) for 15 min at 4°C and washed once with PBS and resuspended in the same buffer, after which the absorbance (A_{600}) of the cell suspension was measured. Equal proportions of viable bacterial culture and solvent (xylene) were then mixed by vortexing for 2 min. A 2-

phase system developed and the aqueous phase was removed for determination of absorbance (A_{600}) after 30 min at room temperature. Affinity to hydrocarbons (hydrophobicity) was reported as the average percentage of 3 replicates, according to the formula BATH% = $[(A_0\text{-}A)/A_0] \times 100$, where A_0 and A are the absorbance before and after mixing with xylene, respectively. The effect of $CaCl_2$, $MgCl_2$, EDTA and Oxgall bile on cell wall hydrophobicity of probiotics was also determined by growing them in MRS media containing $CaCl_2$ (Conc. 0.5, 5.0 and 10.0 mM), $MgCl_2$ (Conc. 0.5, 5.0 and 10.0 mM), EDTA (0.1, 0.3 and 0.5 mM) and Oxgall bile (0.1, 0.3 and 0.5 mM) overnight.

4.2.4 Auto-aggregation analysis

Aggregation of the bacterial cultures was screened using spectrophotometric assays, as described by Collado et al., (2008). Overnight cultures were centrifuged and washed twice with PBS buffer and suspended in the same buffer. Equal volumes of each of the microbial suspensions were combined in sterile test tubes and mixed for 10s on a vortex mixer, and then incubated at 37ºC for 24 h without agitation, anaerobically. The percent aggregation was calculated using the formula $1 - (A_{Time} / A_{initial}) \times 100$, where A_{Time} represents the absorbance of the culture after incubation.

4.2.5 Co-aggregation analysis

The co-aggregation test was performed using bacterial suspensions prepared as described for the auto-aggregation analysis, as follows. Absorbance (A_{600}) of upper suspension was measured after 0, 2, 4, and 24 h of incubation. The co-aggregation (%) was calculated according to the following equation: $[(A_{pat} + A_{probio}) - (A_{mix})/(A_{pat} + A_{probio})] \times 100$, where, A_{pat} and A_{probio}

represent A_{600} of the pathogen (pat) and probiotics (probio) suspensions at time 0 min and A_{mix} represents A_{600} of the mixed bacterial suspension at different times.

Aggregation (auto- and co-aggregation) abilities of micro-organisms were screened by visual observation, and the degree of aggregation was recorded on a scale 0 to 4+ as follows: a score of 0 for no visible aggregates in the cell suspension, 1+ for small uniform aggregates in the suspension, 2+ for aggregates that were easily seen but did not settle, 3+ for large aggregates which settled and left some turbidity in the supernatant fluid, and 4+ for large aggregates which settled and left clear supernatant fluid. Co-aggregation was considered to occur when the score in the reaction mixtures was greater than the auto-aggregation score of either strain in monoculture.

Unstained co-aggregates were also observed under microscope using a 10X dark-field objective lens (0.22 numerical aperture, Carl Zeiss MicroImaging GmbH, Jena, Germany).

4.2.6 Detection of antimicrobial activity of the probiotic supernatant

Extracellular inhibitory substances produced by *L. acidophilus* and present in the culture medium were studied by a modified agar well-diffusion technique (Toure et al., 2003). An overnight MRS culture of *L. acidophilus* grown under standard conditions (section 4.2.1) was centrifuged at 10,000 × g (Sorvall superspeed RC2-B) for 30 min at 4°C and the supernatant was collected. Supernatant was tested for heat stability (100°C for 10 min), and sensitivity to trypsin (EC 3.4.21.4; Sigma Chemical Co., St. Louis, MO) at a final concentration of 1 mg/ml (pH 8.0) at 37°C for 1 h. A 7 mL aliquot of LB or RCM soft agar (0.7% agar) was seeded with 100 µL volume of an overnight culture of the pathogen indicator strain at a final concentration of ~10^7 cfu/mL, and poured on the top of LB or RCM agar plates and allowed to solidify at room temperature. Wells (7 mm) aseptically cut in the solidified agar were filled with either 80 µL of treated or untreated supernatant. The plates were left at 5°C for 2 h to allow diffusion of the

tested supernatant and then incubated aerobically (for *E. coli* O157:H7) or anaerobically (for *Cl. sordelli*) for 24 h at 37°C, after which zones of inhibition were measured.

4.2.7 Growth inhibition assay

Growth inhibition of pathogens by the supernatant of the probiotic was tested using the assay described by Forestier et al. (2001). Briefly, overnight *L. acidophilus* cultures were pelleted at 10,000 × g (Sorvall superspeed RC2-B) for 30 min at 4°C, and the supernatant fluid collected and filtered using a 0.2 μm pore size syringe filter (Fisher Scientific Co., Fair Lawn, NJ) to remove any remaining bacteria. Pathogenic strains to be tested were cultured overnight in their respective media (section 4.2.1). Pathogenic bacteria were harvested by centrifuging at 3,250 × g (Model 5810 R with swing-bucket rotor A-4-81; Eppendorf, Hamburg, Germany) for 10 min at 4°C. The supernatant was discarded and the cells were washed once and resuspended using PBS. The growth inhibition assay was performed by incubating an equal volume of the pathogen culture (containing approximately 1.1×10^8 cfu/mL) along with the probiotic supernatant at 37°C for 24 h. Experiments with *Cl. sordelli* were conducted under anaerobic conditions. MRS broth (pH 6.8 ± 0.2) was used as a control. For both pathogens, aliquots were removed after every 2 h and serially diluted and plated on the appropriate media to quantify the number of surviving bacterial. Control experiments were performed by incubating the same number of pathogens with MRS broth instead of probiotic supernatant. All experiments were conducted in triplicate.

4.3 Results

4.3.1 Agar-spot test

Strong antagonistic activity against the tested pathogen strains was detected by the probiotic strains using the agar-spot method (Figure 4.3.1). All probiotics tested demonstrated inhibition against the enteric pathogens tested. However, *L. acidophilus* showed the most effective inhibition of *E. coli* O157 and *Cl. sordelli* with a zone diameter of 0.8 ± 0.3 cm and 1.4 ± 0.2 cm (Table 4.3.1), respectively, and was selected for further tests to determine its cell surface hydrophobicity, auto-aggregation, co-aggregation and antimicrobial activity with tested pathogen strains.

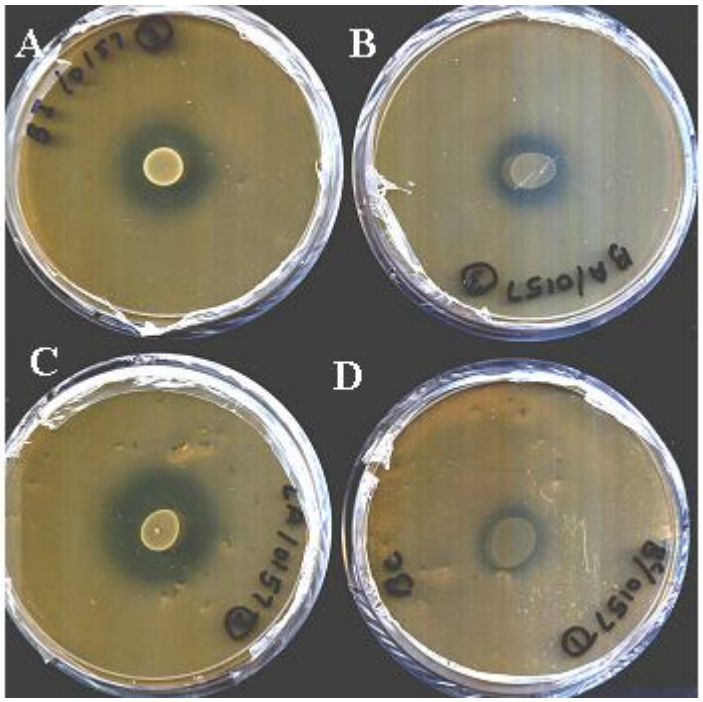

Figure 4.3.1 Antimicrobial activities of probiotics against *E. coli* O157. Agar spot test showing zone of inhibition around probiotic spot. Spots represent: A) *B. infantis;* B) *B. adolescentis;* C) *L. acidophilus;* and D) *B. catenulatum*

Table 4.3.1 Inhibition of selected pathogenic bacteria by probiotics as determined by agar spot test

Pathogens	Probiotics			
	B. infantis	***B. adolescentis***	***L. acidophilus***	***B. catenulatum***
E. coli O157[a]	0.7 ± 0.2	0.6 ± 0.2	0.8 ± 0.3	0.4 ± 0.2
Cl. sordelli[a]	0.4 ± 0.2	0.5 ± 0.1	1.4 ± 0.2	1.0 ± 0.3

[a] Zones of inhibition are expressed as the diameter in cm. The data are expressed as mean ± one standard deviation (n=3).

The diameter of zones of inhibition of *L. acidophilus* was found to be reduced by approximately 50% when 0.2% sodium-bicarbonate (acid control) was added to the MRS agar media (Table 4.3.2 and Figure 4.3.2). The control treatment (MRS media alone) showed no inhibitory effect against the pathogens tested (data not shown).

Table 4.3.2 Antibacterial activities of *L. acidophilus* against selected pathogenic bacteria

Pathogens	Diameter of zones (cm) on MRS	Diameter of zones (cm) on MRS (0.2% Na-carbonate)
E. coli O157[a]	0.8 ± 0.3	0.5 ± 0.2
Cl. sordelli[a]	1.4 ± 0.2	0.7 ± 0.1

[a]The data are expressed as mean ± one standard deviation (n=3).

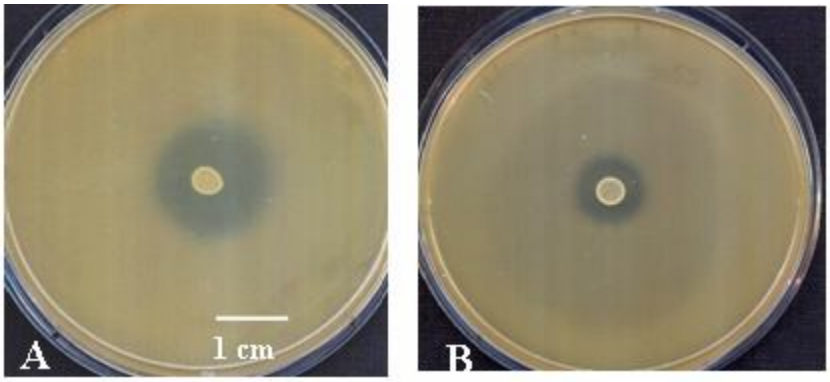

Figure 4.3.2 Agar-spot test showing antibacterial activity of *L. acidophilus* on MRS agar medium overlaid with Luria-Bertani (LB) soft agar seeded with *E. coli* O157:H7. A) MRS (control), and B) MRS containing 0.2% (w/v) sodium-bicarbonate

4.3.2 Bacterial adhesion to hydrocarbons

The percentage of cells adhering to the hydrocarbon xylene was used to provide an index of cell surface hydrophobicity. Percent adhesions of probiotic and pathogen strains to xylene are shown in Table 4.3.3. The most hydrophobic tested pathogen strains were *Aeromonas hydrophila* ATCC 7965, *Citrobacter freundii* ATCC 8090, *Clostridium sordelli* ATCC 9714, *Listeria monocytogenes* ATCC 43256, *Salmonella enteridis* ATCC 4931 and *Yersinia enterocolitica* ATCC 35669. The most hydrophobic probiotic strains were *Lactobacillus acidophilus* ATCC 11975, *Escherichia coli* ATCC 14763, *Escherichia coli* ATCC 4351, *Lactobacillus lactis* ATCC 11954, and *Bifidobacterium catenulatum* ATCC 27675 and *Saccharomyces cerevisiae* ATCC 24859. In general, LAB strains showed a lower hydrophobicity than pathogens. Based on preliminary findings (Table 4.3.1), *L. acidophilus* was further studied for its auto- and co-aggregation properties with *E. coli* O157:H7 and *Cl. sordelli*.

Table 4.3.3 Percent adhesion to hydrocarbons of enteric pathogens and probiotic strains

Pathogen strains	**% BATH**[a]
Aeromonas hydrophilia ATCC 7965	82.0 ± 8.5
Citrobacter freundii ATCC 8090	61.0 ± 2.3
Clostridium perfringens ATCC 13124	41.0 ± 4.9
Cl. sordelli ATCC 9714	66.0 ± 5.5
Enterobacter aerogenes ATCC 13048	30.0 ± 9.1
E. cloacae ATCC 13047	0.0 ± 10.0
Escherichia coli O157:H7	44.5 ± 3.6
Listeria monocytogenes ATCC 43256	96.5 ± 9.1
Salmonella enteridis ATCC 4931	69.0 ± 1.4
Vibrio parahemolyticus ATCC 17802	21.0 ± 12.3
Yeresinia enterocolitica ATCC 35669	84.0 ± 5.6
Yeresinia enterocolitica ATCC 9610	17.0 ± 7.2
Candida albicans ATCC 18804	16.0 ± 8.7
Probiotic strains	
Saccharomyces cerevisiae ATCC 24859	66.0 ± 3.4
L. acidophilus ATCC 11975	46.5 ± 6.1
B. infantis ATCC 15697	2.5 ± 3.6
B. catenulatum ATCC 27675	65.5 ± 6.4
B. adolescentis ATCC 15703	4.0 ± 6.0
Alcaligenes fecalis DSM 30030	-0.07 ± 0.8
Enterococcus fecalis ATCC 19433	18.5 ± 1.4
Enterococcus faecium ATCC 19434	1.0 ± 2.2
Escherichia coli ATCC 14763	55.0 ± 4.7
Escherichia coli ATCC 4351	70.0 ± 7.0
Lactobacillus lactis ATCC 11954	85.0 ± 6.4
Lactobacillus planetarum ATCC 14917	0.0 ± 3.9
Lactobacillus rhamnosus ATCC 7469	-16.6 ± 4.3

[a]The data are expressed as mean ± one standard deviation (n=3).

4.3.3 Bacterial auto-aggregation and co-aggregation analysis

The auto-aggregation properties of *L. acidophilus* and pathogen strains were measured over 24 h at 37°C using absorbance measurements at 600 nm. Results of percentage auto-aggregation are shown in Table 4.3.4. In general, the auto-aggregation abilities of all tested strains increased over a period of 24 h incubation at 37°C. *Clostridium sordelli* was the best auto-aggregating strain among those tested, resulting in 63.6 ± 1.4% auto-aggregation after 24 h incubation.

Table 4.3.4 Adhesion to hydrocarbons as measured using the BATH test and % auto-aggregation of probiotic and pathogenic strains

Bacterial strains	**% Auto-aggregation**[a]		
	2h	4h	24h
L. acidophilus ATCC 11975	7.3 ± 1.0	16.9 ± 2.1	36.7 ± 4.5
E. coli O157:H7	5.2 ± 0.8	15.0 ± 1.0	45.3 ± 7.0
Cl. sordelli ATCC 9714	15.0 ± 6.3	34.0 ± 5.2	63.6 ± 1.4

[a]Mean ± standard deviation of results from three separate experiments.

These results were confirmed by visual aggregation determination; a score of 3+ was given to *Cl. sordelli* and *L. acidophilus* and 2+ was given to *E. coli* auto-aggregates. *Lactobacillus acidophilus* was able to co-aggregate with both tested pathogens, the results of which are shown in Table 4.3.5.

Table 4.3.5 Co-aggregation percentages of pathogen and probiotic strains after incubation at 37ºC, anaerobically

Co-aggregation with *L. acidophilus*	**% Co-aggregation**		
	2h	4h	24h
E. coli O157:H7[a]	16.0 ± 2.4	43.6 ± 1.1	47.9 ± 3.6
Cl. sordelli ATCC 9714[a]	23.2 ± 3.2	43.7 ± 1.5	51.8 ± 4.2

[a]Mean ± standard deviation of results from three separate experiments.

Percent co-aggregation tended to increase over the 24 h incubation period. The percent of co-aggregation of *L. acidophilus* with *E. coli* O157:H7 was 43.6 ± 1.1% after 4 h incubation and 47.9 ± 3.6 % after 24 h. Whereas, *Cl. sordelli* showed 43.7 ± 1.5% co-aggregation after 4 h and 51.8 ± 4.2% after 24 h incubation at 37°C. These results were also confirmed by visual determination (Figure 4.3.3) and microscopic examination (Figure 4.3.4) of co-aggregates. A visual score of 3+ was given to both *E. coli* O157:H7- and *Cl. sordelli-L. acidophilus* co-aggregates.

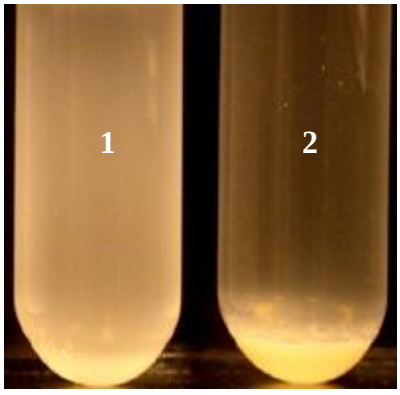

Figure 4.3.3 Visual determination of co-aggregation of *L. acidophilus* ATCC 11975 with *E. coli* O157:H7. Tubes 1 and 2 represents co-aggregation before (0 h), and after (24 h) incubation, respectively

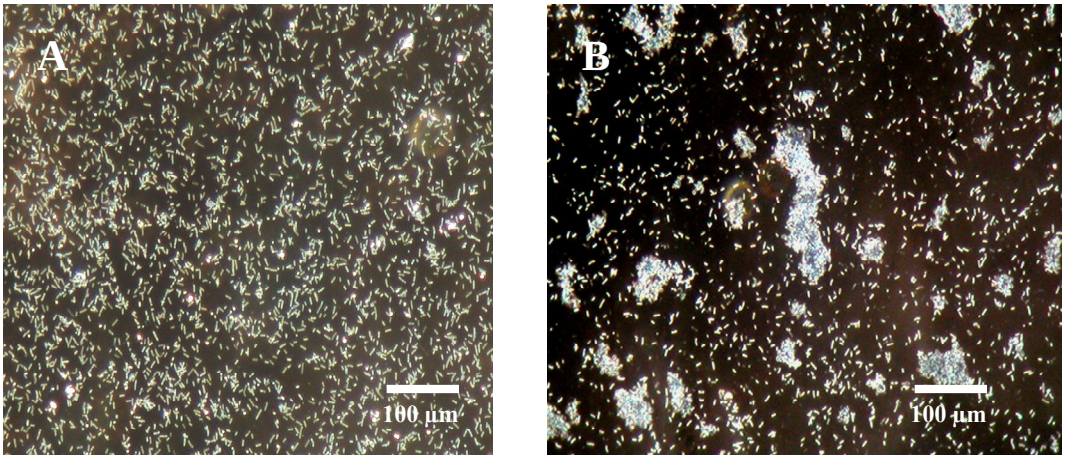

Figure 4.3.4 Dark-field (10X NA 0.22) photomicrographs, showing co-aggregates of *L. acidophilus* and *E. coli* O157. A) Before incubation, and B) Co-aggregates after incubation at 37ºC for 24 h

4.3.4 Detection of antimicrobial activity of probiotic supernatant

Antagonistic activity of *L. acidophilus* supernatant was further evaluated by an agar-well diffusion method. No zones of inhibition were observed with cell-free probiotic supernatant. The antibacterial activity of probiotic cell-free supernatant (pH 4.5 ± 0.1) was examined in broth culture using the Gram-negative aerobe, *E. coli* O157:H7, and then Gram-positive anaerobic pathogen, *Cl. sordelli.* MRS broth (pH 6.7 ± 0.3) was used as control. Standard plate counts were used to determine the viability of the two pathogen strains over a 24 h period (Figure 4.3.5).

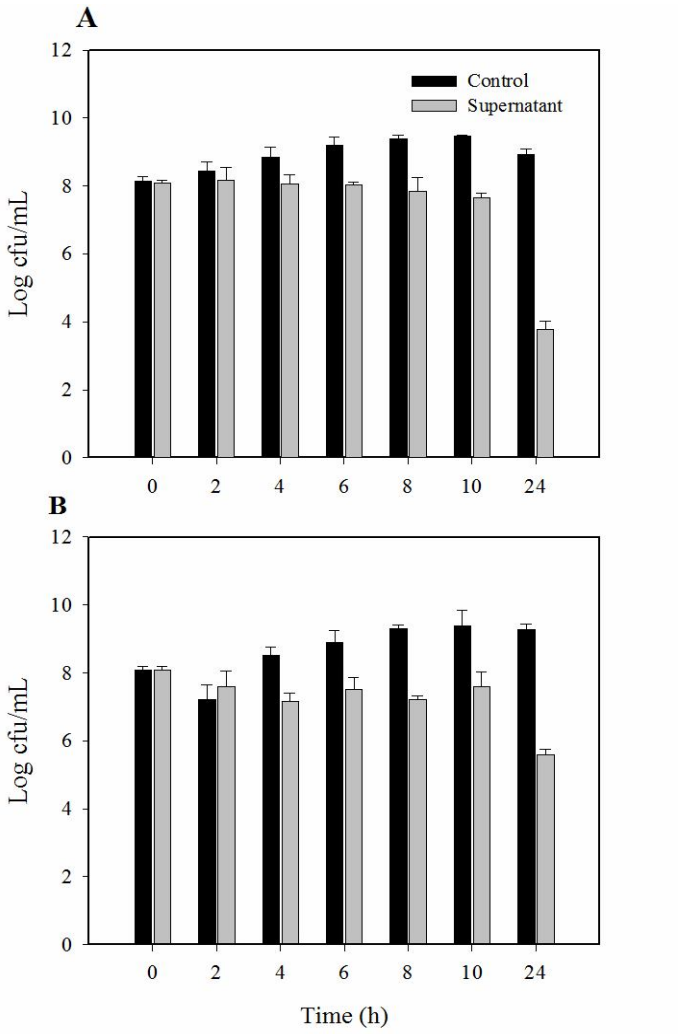

Figure 4.3.5 Antimicrobial activity of *L. acidophilus* supernatant on the growth of A) *E. coli* and, B) *Cl. sordelli*

Results showed that pathogen growth was inhibited, and that none of the pathogen strains grew in the presence of cell-free probiotic supernatant over the first 10 h. After 24 h incubation, the number of viable bacteria was reduced approximately by 4 log and 2 log cfu/ml for *E. coli* O157:H7 and *Cl. sordelli*, respectively, compared to the pH 6.7 ± 0.3 MRS control (no probiotic supernatant; Figure 4.3.5). The treatment of supernatant by trypsin at final concentration of 1mg/ml and heat (100º C for 10 min) did not affect the inhibitory activity (data not shown).

4.4 Discussion

Cell surface hydrophobicity assays do not measure the intrinsic microbial cell surface hydrophobicity, but rather the bacterial partitioning to a certain hydrophobic substrate (i.e., xylene). The BATH test has been extensively used for measuring cell surface hydrophobicity in LAB (Vinderola et al. 2004; Collado et al. 2008). Many studies showed that the presence of (glycol-) proteinaceous material at the cell surface resulted in higher hydrophobicity; whereas, hydrophilic surfaces were associated with the presence of polysaccharides (Rojas et al. 1996). In the present study, the cell surface hydrophobicity and percent auto-aggregation of common enteric pathogens like *Salmonella enteridis* and *Escherichia coli* O157:H7, commensal microflora of GIT including *Escherichia coli* ATCC 14763, *Escherichia coli* ATCC 4351, *Enterococcus fecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, and tested probiotics were determined and are shown in figure A.1 and A.3 (Appendix A). The effect of different cations including $MgCl_2$, $CaCl_2$, EDTA and Oxgall bile on cell surface hydrophobicity of probiotics is evaluated (Figure A.2, Appendix A). Cell surface hydrophobicity was found to be related to auto-aggregation properties as most of the strains with higher adhesion to hydrocarbons showed high auto-aggregation abilities.

Aggregation properties of probiotics with pathogens may be of importance for both food preservation and therapeutic impact on intestinal microbiota. Preliminary screening of co-aggregation potential may be useful to identify potential probiotic strains suitable for food, human or animal use. The present study was in agreement with the findings of Collado et al. (2008) which showed that bacterial strains with higher adhesion to hydrocarbon (xylene) showed high auto-aggregation abilities (Table 4.3.4; Figure A.1 and A.3, Appendix A). *In vitro* analysis of the probiotic *L. acidophilus* demonstrated co-aggregation with both Gram-negative *E. coli* O157:H7 and Gram-positive *Cl.* sordelli (Table 4.3.5). Another important finding of the present

study was that incubation of two probiotic strains with a single pathogen strain resulted in greater co-aggregation and more rapid and complete clearing of liquid in the culture tube (Table A.1 and Figure A.4, Appendix A).

This finding supports the idea that co-aggregation of probiotics with pathogens could potentially interfere with pathogen colonization of the GIT, survival (through various antimicrobial mechanisms) and subsequent manifestation of illness. One hypothesis is that co-aggregation of probiotics with other established commensal GIT microflora may aid in the attachment and colonization of probiotics to mucosal surfaces. The probiotic *L. acidophilus* ATCC 11975 tested in this study showed significant antibacterial activity against both Gram-positive *Cl. sordelli* ATCC 9714 and *Escherichia coli* O157:H7 (Figure 4.3.5). This activity was possibly due to the antibacterial substances produced by this probiotic strain. It has been reported by several investigators that lactobacilli are able to produce antimicrobial substances when grown in specific media; a *L. acidophilus* strain produced lactacin (Barefoot et al., 1983), while a *L. plantarum* isolate produced plantaricin (Anderson et al., 1988). Corr et al. (2007) has demonstrated that the *in vivo* antimicrobial effects of *L. salivarius* UCC118 depended primarily on their capacity to produce the bacteriocin Abp118.

Bacteria generally compete with each other by secreting antagonistic compounds, which may affect cell viability directly or indirectly by modifying the surrounding environment. In the present thesis research, strong probiotic antagonism against the tested pathogen strains was detected using the agar-spot method (Table 4.3.1). Interestingly, the probiotic strain did not exhibit the same antibacterial activity when cell free culture supernatant was tested using the agar-well diffusion method. Similar conflicting results have been reported by Harris et al. (1989), when seven LAB strains produced inhibitory zones against *Listeria monocytogenes* on solid

media, but only three of them produced inhibitory zones via the agar-well diffusion method. The inability to detect antibacterial activity in the probiotic supernatant in the present study may be due to the lower production of this substance; only in close proximity to the producing cells is a threshold concentration achieved necessary to exert an antimicrobial effect. Another possible explanation for the absence of antibacterial activity in the well-diffusion test is that cell-cell contact is necessary between probiotic and pathogen strains for the factor to be produced, something that would be absent when the culture supernatant was used. Accordingly, physical contact between both producer organism (probiotic) and indicator (pathogens) in the same environment might actually be necessary for the induction and synthesis of the antimicrobial. Studies done by Toure et al. (2003) showed that the antibacterial activity of some bifidobacterial strains was recovered by concentrating the spent cultures by speed-vac. The fact that probiotic antagonism against the pathogens did not completely disappear when 0.2% sodium bicarbonate was added to the culture medium (Table 4.3.2; Figure 4.3.2) is strongly suggestive that other antibacterial factors, other than the effect of organic acids, were operative.

Jacobsen et al. (1999) reported that the inhibitory activity of lactobacilli is variable even within the same species. In the present study, the mechanism(s) of pathogen inhibition was seemingly not due to the production of a proteinaceous compound like a bacteriocin, which exhibit antagonistic activity against taxonomically closely-related bacteria (Klaenhammer, 1993; Jack et al., 1995). Previous studies have demonstrated the production of broad-spectrum antibacterial substances, referred to as ’bacteriocin-like’ compounds, by different species of lactobacilli (Silva et al., 1987; Camard et al., 1997). Moreover, treatment of probiotic supernatant with either heat (100°C for 10 min) or trypsin (1 mg/mL for 1 h) failed to eliminate the antagonistic activity of probiotic supernatant during growth inhibition assay (data not

shown). This suggests that the probiotic-dependent inhibitory activity observed in the current study was from the result of a heat stable non-proteinaceous substance. Niku-Paavola et al. (1999) identified various antimicrobial compounds in the culture filtrate of *Lactobacillus plantarum,* including benzoic acid, methylhydantoin and mevalonolactone, that were able to withstand heat treatment at 120°C for 15 min as well as trypsin (1mg/mL for 1 h at 37°C) treatment. However, it was also apparent that production of organic acid, which lowered the pH (4.5 ± 0.1) of the probiotic supernatant, was partially responsible for the observed antagonistic effect. Moreover, the fact that depletion of nutrients in probiotic supernatant could be a possible explanation for growth inhibition of pathogens (figure 4.3.5). The dominance of inhibitor-producing lactobacilli in the gastrointestinal tract and the ability of these organisms to interact closely with enteric pathogens seemingly constitute an important host defense mechanism against infection. Further experiments identify the chemical nature of the antibacterial compound(s), and to establish the inhibition spectrum of the probiotic against a larger panel of pathogenic organisms are necessary. Current findings from this research suggest that *L. acidophilus* ATCC 11975 has the potential for interfering with disease-causing pathogens. Use of GIT model systems to evaluate this inhibitory potential, *in vivo,* would help establish this potential more clearly.

5 General discussion and conclusions

The greatest hurdles that probiotic bacteria must overcome during food processing, storage and in the upper GIT are acid and the presence of bile. A probiotic *L. acidophilus* strain exhibiting acid and bile tolerance has previously been isolated and evaluated. The present characterization studies indicated that *Lactobacillus acidophilus* ATCC 11975 was the most acid-tolerant strain (*D*-value 10.2 ± 0.80 min), able to survive 30 min at pH 2.0, and also bile-

tolerant. Furthermore, the highest adhesion to Caco-2 cells was observed using *L. acidophilus* (4.5×10^4 cfu/mL). *L. acidophilus* has the potential for interfering with disease-causing pathogens including *Escherichia coli* O157:H7 and *Clostridium sordelli* ATCC 9714. Co- and auto-aggregation of *L. acidophilus* with enteric pathogens was also apparent in this study. Use of GIT model systems would help to evaluate the inhibitory potential of *L. acidophilus* more clearly. Knowledge of antimicrobial resistance patterns may also provide important information for consumption of naturally-resistant probiotics during antibiotic therapy, thereby protecting the intestinal mucosa from colonization of deleterious pathogenic strains. Screening for strains that can efficiently utilize certain prebiotics will provide competitive advantage for the probiotics in nutrient deficient large intestine. It is thought that the sole carbon source utilization profiles will provide useful information about the metabolic potential of the different probiotic microorganisms. Results showed that all probiotic strains grew to the highest culture optical densities on D-xylose. Further examination of substrate preferences of probiotics would be necessary for co-delivery of probiotics and prebiotics. In the present study, encapsulation of *L. acidophilus* strains with pea protein-alginate matrix was found to give 6 times more protection under acid stress. Thus future probiotic-prebiotic (synbiotic) combinational developments may be facilitated by screening the potential probiotic strain for desirable characteristics such as probiotic substrate preferences and antibiotic resistance profiling. As a physical delivery tool, delivery of probiotics, with or without prebiotics, within a capsule is suggested to improve the survival and subsequently enhance the growth potential of these beneficial bacteria in the GIT.

6 Recommendations

Further identification and characterization of probiotic cell-wall properties is recommended to understand their role in adhesion to hydrocarbons, auto-aggregation, and relation to co-aggregation mechanisms. Selection of new probiotic combinations should be conducted for specific target pathogens or pathogen-associated microbiota aberrancies. Molecular techniques like multiplex RAPD-PCR, could be used to reveal the complete metabolic potential of each of the probiotic strain which opens the possibility to engineer new combinations of pre- and probiotics.

7 References

Abrahamsson, T. R., Jakobsson, T., Bottcher, M. F., Fredrikson, M., Jenmalm, M. C., and Bjorksten, B., 2007. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. J. Allergy Clin. Immunol., 119: 1174-1180.

Adamowicz, M. P., Kelley, M., and Nickerson, K. W., 1991. Detergent (sodium dodecyl sulfate) shock proteins in *Escherichia coli*. J. Bacteriol., 173: 229-233.

Agarwal, K. N. and Bhasin, S. K., 2002. Feasibility studies to control acute diarrhoea in children by feeding fermented milk preparations, Actimel and Indian Dahi. Eur. J. Clin. Nutr., 56: S56-S59.

Akalin, A. S., Fenderya, S., and Akbulut, N., 2004. Viability and activity of bifidobacteria in yoghurt containing fructooligosaccharide during refrigerated storage. Int. J. Food Sci. Technol., 39: 613-621.

Alakomi, H. L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., and Helander I. M., 2000. Lactic acid permeabilizes Gram negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol., 66: 2001-2005.

Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., and Wright, V. A., 1999. Persistence of colonisation of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG after oral consumption. Appl. Environ. Microbiol., 65: 351–354.

Ammor, M. S., Florez, A. B., Mayo, B., 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol., 24: 559-570.

Anderson, R. E., Daeschel, M. A., and Hassan, H. M., 1988. Antibacterial activity of plantaricin SIK-83, a bacteriocin produced by *Lactobacillus plantarum*. Biochem., 70: 381-390.

Anderson, A. D., McNaught, C. E., Jain, P. K. and MacFie, J., 2004. Randomised clinical trial of symbiotic therapy in elective surgical patients. Gut, 53: 241-245.

Andersson, A. F., Lindberg, M., Jakobsson, H., Backhed, F., Nyren, P., and Engstrand, L., 2008. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS ONE, 3: 1-8.

Apajalahti, J. H. A., Sarkilahti, L. K., Maki, B. R. E., Heikkinen, J. P., Nurminen, P. H., and Holben, W. E., 1998. Effective recovery of bacterial DNA and percent-guanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. Appl. Environ. Microbiol., 64: 4084-4088.

Apajalahti, J. H. A., Kettunen, A., Nurminen, P. H., Jatila, H., and Holben, W. E., 2003. Selective plating underestimates abundance and shows differential recovery of bifidobacterial species from human feces. Appl. Environ. Microbiol., 69: 5731-5735.

Aryana, K., Plauche, J. S., Rao, R. M., Mcgrew, P. and Shah, N. P., 2007. Fat-free plain yogurt manufactured with inulins of various chain lengths and *Lactobacillus acidophilus.* J. food Sci., 72: M79-M84.

Audet, P., Paquin, C., and Lacroix, C., 1988. Immobilized growing lactic acid bacteria with k-carrageenan-locust bean gum gel. Appl. Microbiol. Biotechnol., 29: 11-18.

Axelsson, L. V., 1998. Lactic acid bacteria: classification and physiology. In: Salminen S, Von Wright A, eds. Lactic acid bacteria: microbiology and functional aspects. 2nd ed. Marcel Dekker Inc., New York, 1-72.

Barefoot, S. F., and Klaenhammer, T. R., 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol., 45: 1808-1815.

Begley, M., Hill, C., and Gahan, C. G. M., 2006. Bile Salt Hydrolase Activity in Probiotics. Appl. Environ. Microbiol., 72: 1729-1738.

Beijerinck, M. W., 1901. Lactic acid bacteria of the industry. Arch. Neerl. Sci. Exact. Nat., 6: 212-243.

Benno, I., and Mitsuoka, T., 1992. Impact of *Bifidobacterium longum* on human fecal microflora. Microbiol. Immun., 36: 683-694.

Berg, R. D., 1998. Probiotics, prebiotics or "conbiotics." Trends Microbiol., 6: 89-92.

Bernet, M. F., Brassart, D., Neeser, J. R., and Servin, A. L., 1994. *Lactobacillus acidophilus* LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. Gut, 35: 483-489.

Bertazzoni-Minelli, E., Benini, A., Marzotto, M., Sbarbati, A., Ruzzenente, O., Ferrario, R., Hendriks, H., and Dellaglio, F., 2004. Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional dairy foods. Int. Dairy J., 14: 723–736.

Biavati, B., Castagnoli, P., Crociani, F., and Trovatelli, L. D., 1984. Species of the *Bifidobacterium* in the feces of infants. Microbiologica, 7: 341-345.

Blum, S., and Reniero, R., 2000. Industrial panel statements: adhesion of selected *Lactobacillus* strains to enterocyte-like Caco-2 cells in *vitro:* a critical evaluation of the reliability of in vitro adhesion assays. Proceedings of at the 4th workshop of the PROBEMO-FAIR CT 96-1028 project "Functional Foods for EU health in 2000".

Boever, D. P., Wouters, R., Verschaeve, L., Berckmans, P., Schoeters, G., and Verstraete, W., 2000. Protective effect of the bile salt hydrolase-active *Lactobacillus reuteri* against bile salt cytotoxicity. Appl. Microbiol. Biotecnol., 53: 709-714.

Bomba, A., Nemcova, R., Gancarcikova, S., Herich, R., Guba, P., and Mudronova, D., 2002. Improvement of the probiotic effect of micro-organisms by their combination with maltodextrins, fructo-oligosaccharides and polyunsaturated fatty acids. Br. J. Nutri., 88: S95-S99.

Bonaparte, C., 1997. Selective isolation and taxonomic position of bifidobacteria isolated from commercial fermented dairy products in central Europe. Dissertation. Technische Universitat, Berlin.

Boris, S., Suarez, J. E., Vazquez, F., and Barbes, C., 1998. Adherence of human vaginal *Lactobacilli* to vaginal epithelial cells and interaction with uropathogens. Infect. Immun., 66: 1985-1989.

Bouhnik, Y., Attar, A., Joly, F. A., Riottot, M., Dyard, F., and Flourie, B., 2004. Lactulose ingestion increases faecal bifidobacterial counts: A randomised double-blind study in healthy humans. Euro. J. Clin. Nutri., 58: 462-466.

Bouhnik, Y., Kvahedi, L., Achour, Attar, A., Salfati, J., Pochart, P., Marteau, P., Flourie, B., Bornet, F., and Rambaud, J. C., 1999. Short chain fructo-oligosaccharide administration dose-dependently increases fecal bifidobacteria in healthy humans. J. Nutr., 129: 113-116.

Bruzzese, E., Volpicelli, M., Squaglia, M., Tartaglione, A., and Guarino, A., 2006. Impact of prebiotics on human health. Digest. Liver Dis., 38: S283-S287.

Bunthof, C. J., Bloemen, K., Breeuwer, P., Rombouts, F. M., and Abee, T., 2001. Flow Cytometric Assessment of Viability of Lactic Acid Bacteria. Appl. Environ. Microbiol., 67: 2326-2335.

Camard, M. F. B., Brassart, V. L. B., Neeser, J. R., Servin, A. L., and Hudault, S., 1997. The human *Lactobacillus acidophilus* Strain LA1 secretes a non-bacteriocin antibacterial substance(s) active in vitro and in vivo. Appl. Environ. Microbiol., 63: 2747-2753.

Capela, P., Hay, T. K. C., and Shah, N. P., 2006. Effect of cryoproyectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. Food Res. Int., 39: 203-211.

Cebra, J. J., 1999. Influences of microbiota on intestinal immune system development. Am. J. Clin. Nutr., 69: 1046S-1051S.

Cereijido, M., Valdes, J., Shoshani, L. and Contreras, R. G., 1998. Role of tight junctions in establishing and maintaining cell polarity. Annu. Rev. Physiol. 60: 161-177.

Chandramouli, V., Kailasapathy, K., Peiris, P., and Jones, M., 2004. An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. J. Microbiol. Meth., 56: 27– 35.

Charalampopoulos, D., Pandiella, S. S. and Webb, C., 2003. Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. Int. J. Food Microbiol., 82: 133–141.

Charteris, W. P., Kelly, P. M., Morelli, L., and Collins, J. K., 1998a. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Microbiol. 84: 759-768.

Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K., 1998b. Antibiotic susceptibility of potentially probiotic *Bifidobacterium* isolates from the human gastrointestinal tract. Lett. Appl. Microbiol., 26: 333–337.

Chauviere, G., Coconnier, M. H., Kerneis, S., Fournait, J. and Servin, A. L., 1992a. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. J. Gen. Microbiol., 138: 1689-1696.

Chauviere, G., Coconnier, M. H., Kerneis, S., Michaud, A. D., Joly, B., and Servin, A. L., 1992b. Competitive exclusion of diarrheagenic *Escherichia coli* (ETEC) from human enterocyte-like Caco-2 cells by heat-killed *Lactobacillus*. FEMS Microbiol. Lett., 70: 213-217.

Choi, W. S. and Han J. A., 2002. Film-forming mechanism and heat denaturation effects on the physical and chemical properties of pea-protein-isolate edible films. J. Food Sci., 67:1399-1406.

Christiaens, H., Leer, R. J., Pouwels, P. H., and Verstraete, W., 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. Appl. Environ. Microbiol., 58: 3792-3798.

Coconnier, M. H., Bernet, M. F., Chauviere, G., and Servin, A. L., 1993. Adhering heat-killed human *Lactobacillus acidophilus*, strain LB inhibits the process of pathogenicity of diarrhoeagenic bacteria in cultured human intestinal cells. J. Diarrhoeal Dis. Res., 11: 235-242.

Collado, M. C., Meriluoto, J., and Salminen, S., 2007a. Development of new probiotics by strain combinations: Is it possible to improve the adhesion to intestinal mucus? J. Dairy Sci., 90: 2710-2716.

Collado, M. C., Surono, I., Meriluoto, J., and Salminen, S., 2007b. Indigenous dadih lactic acid bacteria: Cell-surface properties and interactions with pathogens. J. Food Sci., 72: M89-M93.

Collado, M. C., Meriluoto, J., and Salminen, S., 2008. Adhesion and aggregation properties of probiotic and pathogen strains. Eur. Food Res. Technol., 226: 1065-1073.

Collins, M. D., Rodrigues, U., and Ash, C., 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett.; 77: 5-12.

Collins, J. K., Thornton, G., and Sullivan, G. O., 1998. Selection of probiotic strains for human application. Int. Dairy J., 8: 487-490.

Conway, P. L., Gorbach, S. L., and Golden, B. R., 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci., 70: 1-12.

Corcoran, B. M., Stanton, C., Fitzgerald, G. F., and Ross R. P., 2005. Survival of probiotic Lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. Appl. Environ. Microbiol., 71: 3060-3067.

Corr, S. C., Li, Y., Riedel, C. U., Toole, P., Hill, C., and Gahan, C. G. M., 2007. Bacteriocin production as a mechanism for the anti-infective activity of *Lactobacillus salivarius* UCC118. PNAS, 104: 7617–7621.

Cummings, J. H., Christie, S., and Cole, T. J., 2001. A study of fructo-oligosaccharides in the prevention of traveller's diarrhoea. Aliment. Pharmacol. Ther., 15: 1139-1145.

Cusick, S. M., and O'Sullivan, D. J., 2000. Use of a single, triplicate arbitrarily primed-PCR procedure for molecular fingerprinting of lactic acid bacteria. Appl. Environ. Microbiol., 66: 2227-2231.

Dahiya, R. S., and Speck, M. L., 1967. Hydrogen peroxide formation by *Lactobacilli* and its effect on *Staphylococcus aureus*. J. Dairy Sci., 51: 1568p-1572.

Danone, 2001. Fermented Foods and Healthy Digestive Functions. France: Danone Publications, John Libbey Eurotext.

Dave, N., and Shah, P., 1997. Viability of yogurt and probiotic bacteria in yogurts made from commercial starter culture. Int. Dairy J., 731-741.

De Keersmaecker, S. C., Verhoeven, T. L., Desair, J., Marchal, K., Vanderleyden, J. and Nagy, I., 2006. Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. FEMS Microbiol. Lett., 259: 89-96.

De Vrese, M., Stegelmann, A., Richter, B., Fenselau, S., Laue, C., and Schrezenmeir, J., 2001. Probiotics - compensation for lactase insufficiency. Am. J. Clin. Nutr., 73: 421S-429S.

Defoirdt, T., Boon, N., Bossier, P., and Verstraete, W., 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. Aqua., 240: 69-88.

Del Re, B., Sgorbati, B., Miglioli, M., and Palenzona, D., 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Lett. Appl. Microbiol. 31: 438-442.

Desai, A. R., Musil, K. M., Carr, A. P., and Hill, J. E., 2009. Characterization and quantification of feline fecal microbiota using cpn60 sequence-based methods and investigation of animal-to-animal variation in microbial population structure. Vet. Microbiol., 137: 1-9.

Desmond, C., Ross, R. P., OCallaghan, E., Fitzgerald, G. and Stanton, C., 2002. Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. J. Appl. Microbiol., 93: 1003-1011.

Didierlaurent, A., Sirard, J. C., Kraehenbuhl, J. P., and Neutra, M. R., 2002. How the gut senses its content? Cell. Microbiol., 4: 61-72.

Dirix, G., Monsieurs, P., Dombrecht, B., Daniels, R., Marchal, K., Vanderleydena, J., and Michiels, J., 2004. Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters. Peptides, 25: 1425-1440.

Doderlein, A., 1892. The vaginal transudate and its significance for childbed fever. Centralbl. Bakteriol., 11: 699-700.

Doern, G. V., Dascal, A., and Keville, M., 1985. Susceptibility testing with the sensititer breakpoint broth microdilution system. Diagn. Microbiol. Infect. Dis., 3: 185-191.

Dong, Y. H., Gusti, A. R., Zhang, Q., Xu, J. L., and Zhang, L. H., 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Appl. Environ. Microbiol., 68: 1754-1759.

Drider, D., Fimland, G., Chard, Y., McMullen, L. M., and Prevost, H., 2006. The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev., 70: 564–582.

Dumonceaux, T. J., Hill, J. E., Briggs, S. A., Amoako, K. K., Hemmingsen, S. M., and Van Kessel, A. G., 2006. Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the chaperonin-60 target. J. Microbiol. Meth., 64: 46-62.

Dunne, C., OMahony, L., Murphy, L., Thornton, G., Morrissey, D., OHalloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G. C., Shanahan, F., and Collins, J. K., 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. Am. J. Clin. Nutr., 73: 386S-392S.

Elliott, S. E., Buret, A., McKnight, W., Miller, M. J. S., and Wallace, J. L., 1998. Bacteria rapidly colonize and modulate healing of gastric ulcers in rats. Am. J. Physiol., 275: G425-G432.

Erkkila, S., and Petaja, E., 2000. Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Sci., 55: 297-300.

Escherich, T., 1885. Die darmbakterien des neugeborenen und sauglings. Fortschr. Med., 3: 515-522.

Favaro-Trindale, C. S. and Grosso, C. R. F., 2002. Microencapsulation of *L. acidophilus* (La-05) and *B. lactis* (Bb-12) and evaluation of their survival at the pH values of the stomach and in bile. J. Microencap., 19: 485-494.

Favier, C., Neut, C., Mizon, C., Cortot, A., Colombel, J. F., and Mizon, J., 1997. Faecal β-D-galactosidase production and bifidobacteria are decreased in Crohn's-disease. Digest. Dis. Sci., 42: 817-822.

Fayol-Messaoudi, D., Berger, C. N., Coconnier-Polter, M. H., Lievin-Le Moal, V. and Servin, A. L., 2005. pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic lactobacilli against *Salmonella enterica* serovar *typhimurium*. Appl. Environ. Microbiol., 71: 6008-6013.

Fernandes, C. F., Shahani, K. M., and Amer, M. A., 1987. Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products. FEMS Microbiol. Rev., 46: 343–356.

Finegold, S. M., Sutter, V. L., Sugihara, P. L., Elder, H. A., Lehmann, S. M., and Phillips, R. L., 1977. Fecal microbial flora in seventh day adventist populations and control subjects. Am. J. Clin. Nutr., 30: 1781-1792.

Food and Agriculture Organization and World Health Organization (FAO/WHO), 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. FAO and WHO Joint Expert Committee Report.

Food and agricultural organization and World health Organization (FAO/WHO), 2002. Joint working group report on drafting guidelines for the evaluation of probiotics in food. London, Ontario, April 30 and May 1.

Forestier, C., Champs, C. D., Vatoux, C., and Joly, B., 2001. Probiotic activities of *Lactobacillus casei rhamnosus*: in vitro adherence to intestinal cells and antimicrobial properties. Res. Microbiol., 152: 167-173.

Franz, C. M. A. P., Specht, I., haberer, P. and Holzapfel, W. H., 2001. Bile salt hydrolase activity of *Enterococci* isolated from food: screening and quantitative determination. J. Food Prot., 64: 725-729.

Fredrickson, A. G. and Stephanopoulos, G., 1981. Microbial competition. Science, 213: 972–979.

FUFOSE working group, 1999. Scientific concepts of functional foods in Europe- Consensus document. Br. J. Nutr., 81: S1-S27.

Fuller, R., 1975. Nature of the determinant responsible for the adhesion of lactobacilli to chicken crop epithelial cells. J. Gen. Microbiol., 87: 245-250.

Fuller, R., 1989. Probiotics in man and animals. J. Appl. Bacteriol., 66: 365–378.

Gallaher, D. D., and Khil, J., 1999. The effect of synbiotics on colon carcinogenesis in rats. J. Nutr., 129: 1483S-1487S.

Gan, B. S., Kim, J., Reid, G., Cadieux, P., and Howard, J. C., 2002. *Lactobacillus fermentum* RC-14 inhibits *Staphylococcus aureus* infection of surgical implants in rats. J. Infect. Dis., 185: 1369-1372.

Gardiner, G. E., Ross, R. P., Wallace, J. M., Scanlan, F. P., Jagers, P. P., Fitzgerald, F. P., Collins, J. K., and Stanton, C., 1999. Influence of a probiotic adjunct culture of *Enterococcus faecium* on the quality of cheddar cheese. J. Agric. Food Chem., 47: 4907-4916.

Gardiner, G. E, Heinemann, C., Baroja, M. L., Bruce, A. W., Beuerman, D., Madrenas, J., Reid, G., 2002. Oral administration of the probiotic combination *Lactobacillus rhamnosus* GR-1 and *L. fermentum* for human intestinal applications. Int. Dairy J., 12: 191-196.

GastroNet Australia, 2001. Your digestive system, downloaded from http://www.gastro.net.au/frame_digestive.html on 14.06.2001.

Gauthier, R., 2002. Intestinal health, the key to productivity (The case of organic acids). Precongreso Cientifico Avicola IASA, XXVII Convencion ANECA-WPDC, Puerto Vallarta, Jal. Mexico, 1-12.

Gibson, G. R., and Roberfroid, M. R., 1995. Dietary modulation of the human colonic microbial: introducing the concept of prebiotics. J. Nutr., 125: 1401-12.

Gilliland, S. E., Staley, T. E., and Bush, L. J., 1984. Importance in bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. J. Dairy Sci., 67: 3045-3051.

Goktepe, I., Juneja, V. K. and Ahmedna, M., 2006. Probiotics in food safety and human health. Boca Raton, FL: Taylor and Francis group.

Goldin, B. R., Gorbach, S. L., Saxelin, M., Barakat, S., Gualtieri, L., and Salminen, S., 1992. Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. Dig. Dis. Sci., 173: 121-128.

Gombotz, W. R., and Wee, S. F., 1998. Protein release from alginate matrices. Adv. Drug Deliv. Rev., 31: 276-285.

Gomes, A. M. P., and Malcata, F. X., 1999. *Bifidobacterium* species and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. Trends Food Sci. Technol., 10: 139-157.

Gopal, A., Shah, N. P. and Roginski, H., 1996. Bile tolerance, taurocholate deconjugation and cholesterol removal by *Lactobacillus acidophilus* and *Bifidobacterium* spp. Milchwissenschaft., 51: 619-623.

Gopal, P. K., Prasad, J., Smart, J., and Gill, H. S., 2001. *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. Int. J. Food Microbiol., 67: 207-216.

Grill, J. P., Perrin, S., and Schneider, F., 2000a. Bile salt toxicity to some *Bifidobacteria* strains: role of conjugated bile salt hydrolase and pH. Can. J. Microbiol., 46: 878-884.

Grill, J. P., Cayuela C., Antonie, J. M., and Schneider, F., 2000b. Isolation and characterization of a *Lactobacillus amylovorus* mutant depleted in conjugated bile salt hydrolase activity: relation between activity and bile salt resistance. J. Appl. Microbiol., 89: 553-563.

Groboillot, A. F., Champagne, C. P., Darling, G. D., and Poncelet, D., 1993. Membrane formation by interfacial cross-linking of chitosan for microencapsulation of *Lactococcus lactis*. Biotechnol. Bioeng., 42: 1157-1163.

Gueimonde, M., Margolles, A., Reyes-Gavilan, C. G., and Salminen, S., 2007. Competitive exclusion of enteropathogens from human intestinal mucus by *Bifidobacterium* strains with acquired resistance to bile: a preliminary study. Int. J. Food Microbiol., 113: 228-232.

Harris, L. J., Daeschel, M. A., Stiles, M. E., and Klaenhammer, T. R., 1989. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. J. Food Protect., 52: 384-387.

Havenaar, R., and Veld, J. H. J., 1992. Probiotics, a general view. *In*: B. B. Wood (Ed.) The lactic acid bacteria in health and disease. Elsevier, London, 1: 209-224.

Heatley, R. V., and G. M. Sobala. 1993. Acid suppression and gastric flora. Baillere's Clin. Gastroenterol., 7: 167-181.

Hertzler, S. R., and Savaiano, D. A., 1996. Colonic adaptation to daily lactose feeding in lactose maldigesters reduces lactose intolerance. Am. J. Clin. Nutr., 64: 232-236.

Hofmann, A. F., and Roda, A., 1984. Physicochemical properties of bile acids and their relationship to biological properties: an overview of the problem. J. Lipid Res., 25: 1477–1489.

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U. and Huis in't Veld, J. H., 1998. Overview of gut flora and probiotics. Int. J. Food Microbiol., 41: 85-101.

Holzapfel, W. H., Haberer, P., Geisen, R., Bjorkroth, J., and Schillinger, U., 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr., 73: 365S-73S.

Holzapfel, W. H., and Schillinger, U., 2002. Introduction to pre- and probiotics. Food Res. Int., H35: 109-116.

Hood, S. K., and Zottola, A., 1988. Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. J. Food. Sci., 53: 1514-1516.

Hopkins, M. J., Cummings, J. H. and Macfarlane, G. T., 1998. Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. J. Appl. Microbiol., 85: 381-386.

Hou, R.C., Lin M. Y., Wang, M. M. and Tzen, J. T., 2003. Increase of viability of entrapped cells of *Lactobacillus delbrueckii* spp. *Bulgaricus* in artificial sesame oil emulsions. J. Dairy Sci., 86: 424-428.

Jack, R. W., Tagg, J. R., and Ray, B., 1995. Bacteriocins of Gram-positive bacteria. Microbiol. Rev., 59: 171-200.

Jacobs, M. F., Tynkkynen, S., and Sibakov, M., 1995. Highly bioluminescent *Streptococcus thermophilus* strain for the detection of dairy relevant antibiotics in milk. Appl. Microbiol. Biotecnol., 44: 405.

Jacobsen, C. N., Nielsen, V. R., Hayford, A. E., Moller, P. L., Michaelsen, K. F., Paerregaard, A., Sandstrom, B., Tvede, M. and Jakosen, M., 1999. Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Appl. Environ. Microbiol., 65: 4949-4956.

Jin, L. Z., Marquardt, R. R. and Baidoo, S. K., 2000. Inhibition of enterotoxigenic *Escherichia coli* K88, K99 and 987P by the *Lactobacillus* isolates from porcine intestine. J. Sci. Food Agric., 80: 619-624.

Kailasapathy, K. and Chin, J., 2000. Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. Immunol. Cell Biol., 78: 80-88.

Kalliomaki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., and Isolauri, E., 2001. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet, 357: 1076-1079.

Kawamoto, K., Horibe, I., and Uchida, K., 1989. Purification and characterization of a new hydrolase for conjugated bile acids, chenodeoxy- cholyltaurine hydrolase, from *Bacteroides vulgatus*. J. Biochem., 106: 1049-1053.

Kebary, K. M. K., Hussein, S. A., and Badawi, R. M., 1998. Improving viability of *Bifidobacteria* and their effect on frozen ice milk. Egypt. J. Dairy Sci., 26: 319-337.

Kets, E. P. W., Teunissen, P. J. M., and Bont, J. A. M., 1996. Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. App. Environ. Microbiol., 62: 259-291.

Kim, K. I., and Yoon, Y. H., 1995. A study on the preparation of direct vat lactic acid bacterial starter. Korea. J. Dairy Sci., 17: 129-134.

Kim, S., Cho, S. Y., Kim, S. H., Song, O., Shind, S., Cha, D., and Parke, H. J., 2008. Effect of microencapsulation on viability and other characteristics in *Lactobacillus acidophilus* ATCC 43121. LWT, 41: 493-500.

Kimura, K., McCartney, A. L., McConnell, M. A. and Tannock, G. T., 1997. Analysis of fecal populations of bifidobacteria and lactobacilli and investigation of the immunological

responses of their human hosts to the predominant strains. Appl. Environ. Microbiol., 63: 3394-3398.

Klaenhammer, T. R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev., 12: 39–85.

Klare, I., Konstabel, C., Bertling, S. M., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., and Witte, W., 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of *Lactobacilli, Pediococci, Lactococci,* and *Bifidobacteria*. Appl. Environ. Microbiol., 71: 8982-8986.

Klein, G., Pack, A., Bonaparte, C., and Reuter, G., 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food. Microbiol., 41: 103-125.

Kolars, J. C., Levitt, M. D., Aouji, M., and Savaiano, D. A., 1984. Yoghurt- an auto digesting source of lactose. N. Engl. J. Med., 310: 1-3.

Kollath, W., 1953. Ernahrung und Zahnsystem. Deutsche Zahnarztliche Zeitschrift. 8: 7-16.

Kopp-Hoolihan, L., 2001. Prophylactic and therapeutic uses of probiotics: a review. J. Am. Diet Assoc., 101: 229-238.

Krasaekoopt, W., Bhandari, B., and Deeth, H. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. Int. Dairy J., 13: 3-13.

Kurdi, P., Veen, H. W. V., Tanaka, H., Mierau, I., Konings, W. N., Tannock, G. W., Tomita, F., and Yokota, A., 2000. Cholic acid is accumulated spontaneously, driven by membrane delta pH, in many lactobacilli. J. Bacteriol., 182: 6525-6528.

Lambert, R. J., and Stratford, M., 1999. Weak-acid preservatives: modelling microbial inhibition and response. J. Appl. Microbiol., 86: 157–164.

Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., and Kamphuis, G. R., Wilkinson, M. H. and Welling, G. W., 1995. Quantitative fluorescence *in situ* hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in faecal samples. Appl. Environ. Microbiol., 61: 3069-3075.

Lankaputhra, W. E. V., and Shah, N. P., 1995. Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp in the presence of acid and bile salts. Cult. Dairy Prod. J., 30: 2-7.

Lee, K. I., and Heo, T. R., 2000. Survival of *Bifidobacterium longum* immobilized in calcium alginate beads in simulated gastric juices and bile salt solution. Appl. Environ. Microbiol., 66: 869-973.

Levri, K. M., Ketvertis, K., Deramo, M., Merenstein, J. H., and D'Amico F., 2005. Do probiotics reduce adult lactose intolerance? A systematic review. J. Fam. Pract., 54: 613-620.

Lian, W. C., Hsiao, H. C., and Chou, C. C., 2003. Viability of microencapsulated bifidobacteria in simulated gastric juice and bile solution. Int. J. Food Microbiol., 86: 293-301.

Lidbeck, A., and Nord, C. E., 1993. Lactobacilli and the normal human anaerobic microflora. Int. J. Tissue React., 13: 115-122.

Lilly, D. M., and Stillwell, R. H., 1965. Probiotics: growth promoting factors produced by microorganisms. Science, 147: 747-748.

Ling, W. H., Korpela, R., Mykkanen, H., Salminen, S. and Hanninen, O., 1994. *Lactobacillus* strain GG supplementation decreases colonic hydrolytic and reductive enzyme activities in healthy female adults. J. Nutr., 124: 18-23.

Lipski, A., Friedrich, U., and Altendorf, K., 2001. Application of rRNA-targeted oligonucleotide probes in biotechnology. Appl. Microbiol. Biotechnol., 56: 40-57.

Liu, Q., Duan, Z. P., Ha da, K., Bengmark, S., Kurtovic, J., and Riordan, S. M., 2004. Synbiotic modulation of gut flora: effect on minimal hepatic encephalopathy in patients with cirrhosis. Hepatol., 39: 1441-1449.

Long, S. S., and Swenson, R. M., 1977. Development of anaerobic fecal flora in healthy new-born infants. J. Pediatr., 91: 298–301.

Losada, M. A., and Olleros, T., 2002. Towards a healthier diet for the colon: the influence of fructooligosaccharides and lactobacilli on intestinal health. Nutri. Res., 22: 71-84.

Lourens-Hattingh, A., and Viljoen, B. C., 2001. Review: Yoghurt as probiotic carrier food. Int. Dairy J., 11: 1-17.

Lundeen, S. G., and Savage, D. C., 1990. Characterization and purification of bile salt hydrolase from *Lactobacillus* spp. strain 100-100. J. Bacteriol., 172: 4171-4177.

Macfarlane, G. T., Steed, H., and Macfarlane, S., 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J. Appl. Microbiol., 104: 305-344.

Madureira, A. R., Pereiraa, C. I., Truszkowskab, Gomesa, A. M., Pintadoa, M. E., and Malcataa, F. X., 2005. Survival of probiotic bacteria in a whey cheese vector submitted to environmental conditions prevailing in the gastrointestinal tract. Int. Dairy J., 15: 921-927.

Makras, L., Triantafyllou, V., Fayol-Messaoudi, D., Adriany, T., Zoumpopoulou, G. and Tsakalidou, E., 2006. Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar *typhimurium* reveals a role for lactic acid and other inhibitory compounds. Res. Microbiol., 157: 241-247.

Margolles, A., Garcia, L., Sanchez, B., Gueimonde, M., and Gavilan, C. G., 2003. Characterization of a *Bifidobacterium* strain with acquired resistance to cholate - A preliminary study. Int. J. Food Microbiol., 82: 191-198.

Masco, L., Hoorde1, K. V., Brandt, E. D., Swings, J., and Huys, G., 2006. Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products. J. Antimicrob. Chemother., 58: 85-94.

Matijasic, B. B., Narat, M., and Zoric, M., 2003. Adhesion of two *Lactobacillus gasseri* probiotic strains on Caco-2 cells. Food Tech. Biotechnol., 41: 83-88.

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., Oyaizu, H., and Tanaka, R., 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol., 68: 5445-5451.

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S. A. B., and Williams, P., 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiol., 143: 3703-3711.

Metchnikoff, E., 1907. The prolongation of life. Optimistic studies. William Heinemann, London. United Kingdom.

Miller, M. B., Bassler, B. L., 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol., 55: 165-199.

Millette, M., Luquet, F. M. and Lacroix, M. 2007. *In vitro* growth control of selected pathogens by *Lactobacillus acidophilus* and *Lactobacillus casei*-fermented milk. Lett. Appl. Microbiol., 44: 314–319.

Minekus, M., Marteau, P., Havenaar R, and Huisintveld, J. H. J., 1995. A multicompartmental dynamic computer-controlled model simulating the stomach and small-intestine. ATLA Alternat. Lab Animals. 23: 197-209.

Mitsuoka, T., 1990. Bifidobacteria and their role in human health. J. Ind. Microbiol., 6: 263-268.

Molin, G., Jeppsson, B., and Johansson, M. L., 1993. Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. J. Appl. Bacteriol., 74: 314-23.

Moore, W. E. C., Cato, E. P., and Holdeman, L. V., 1978. Some current concepts in intestinal bacteriology. Am. J. Clin. Nutr., 31: S33-S42.

Morelli, L., 2000. *In vitro* selection of probiotic lactobacilli: a critical appraisal. Curr. Iss. Intest. Microbiol., 1: 59-67.

Morelli, L., 2007. *In vitro* assessment of probiotic bacteria: From survival to functionality. Int. Dairy J., 17: 1278-1283.

Moro, E., 1900. *Bacillus acidophilus* n. spec., A contribution to the knowledge of the normal intestinal bacteria of infants. Jahr. Kinderheilkunde, 52: 38-55.

Mortazavian, A., Razavi, S. H., Ehsani, M. R., and Sohrabvandi, S., 2007. Principles and methods of microencapsulation of probiotic microorganisms. Iran. J. Biotech., 5: 1-18.

Moser, S. A., and Savage, D. C., 2001. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in *Lactobacilli*. Appl. Environ. Microbiol., 67: 3476-3480.

Moubareck, C., Gavini, F., Vaugien, L., Butel, M. J. and Doucet-Populaire, F., 2005. Antimicrobial susceptibility of bifidobacteria. J. Antimicrob. Chemother., 55: 38-44.

Naaber, P., Smidt, I., Stsepetova, J., Brilene, T., Annuk, H. and Mikelsaar, M., 2004. Inhibition of *Clostridium difficile* strains by intestinal *Lactobacillus* species. J. Med. Microbiol., 53: 551–554.

National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial susceptibility testing: Twelfth informational supplement M100-S12. National committee for clinical laboratory standards, Villanova.

National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard M31-A2. National committee for clinical laboratory standards, Villanova.

Nealson, K. H., Platt, T., and Hastings, J. W., 1970. Cellular control of the synthesis and activity of the bacterial luminescence system. J. Bacteriol., 104: 313- 322.

Nicaise, P., Gleizes, A., Sandre, C., Kergot, R., Lebrec, H., Forestier, F. and Labarre, C., 1999. The intestinal microflora regulates cytokine production positively in spleen-derived macrophages but negatively in bone marrow-derived macrophages. Eur. Cytokine Netw. 10: 365-372.

Niku-Paavola, M. L., Laitila, A., Mattila-Sandholm, T. and Haikara, A., 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J. Appl. Microbiol., 86: 29-35.

Noriega, L., Gueimonde, M., Sanchez, B., Margolles, A., Clara, G., and Reyes-Gavilan, D. L., 2004. Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low PH and cross-resistance to bile salts in Bifidobacterium., Int. J. Food Microbiol., 94: 79-86.

Northfield, T. C., and McColl, I., 1973. Postprandial concentrations of free and conjugated bile acids down the length of the normal human small intestine. Gut, 14: 513-518.

O'Sullivan, M. G., Thornton, G. M., O'Sullivan, G. C., and Collins, J. K., 1992. Probiotic bacteria: myth or reality? Trends Food Sci. Technol., 3: 309-14.

Ocana, V. S. Holgado, A. P. R., and Macias, M. E. N., 1999. Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius s*train. Appl. Environ. Microbiol., 65: 5631-5635.

Oh, S., Kim, S. H., and Worobo, R. W., 2000. Characterization and purification of a bacteriocin produced by a potential probiotic culture, *Lactobacillus acidophilus* 30SC. J Dairy Sci., 83: 2747–2752.

Oppegard, C., Fimland, G., Thorbaek, L., and Meyer, J. N., 2007. Analysis of the two-peptide bacteriocins Lactococcin G and Enterocin 1071 by site-directed mutagenesis. Appl. Environ. Microbiol., 73: 2931–2938.

Otero, M. C., and Macias, M. E. N., 2006. Inhibition of *Staphylococcus aureus* by H_2O_2-producing *Lactobacillus gasseri* isolated from the vaginal tract of cattle. Anim. Reprod. Sci., 96: 35-46.

Ouwehand, A., Salminen, S., and Isolauri, E. 2002. Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek, 82: 279-289.

Ozer, B., Kirmaci, H. A., Senel, E., Atamer, M., and Hayaloglu. A., 2009. Improving the viability of *Bifidobacterium bifidum* BB-12 and *Lactobacillus acidophilus* LA-5 in white-brined cheese by microencapsulation., Int. Dairy J., 19: 22-29

Palframan, R., Gibson, G. R., Rastall, R. A., 2003. Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. Lett. Appl. Microbiol., 37: 281-284.

Parvez, S., Malik, K. A., Kang, S. A., and Kim, H. Y., 2006. Probiotics and their fermented food products are beneficial for health. J. Appl. Microbiol., 100: 1171–1185.

Pennacchia, C., Vaughan, and Villani, F., 2006. Potential probiotic *Lactobacillus* strains from fermented sausages: Further investigations on their probiotic properties. Meat Sci., 73: 90-101.

Perdigon, G., Galdeano, M. C., Valdez, J. C., Medici, M., 2002. Interaction of lactic acid bacteria with the gut immune system. Eur. J. Clin. Nutr., 4: S21-26.

Pierucci, A. P. T. R., Andrade, L. R., Farina, M., Pedrosa, C., and Rocha-Leao M. H. M., 2007. Comparison of α-tocopherol microparticles produced with different wall materials: pea protein a new interesting alternative. J. Microencap., 24: 201-213.

Pleiss, E. M., D., and Dicks, L. M. T., 1995. Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus gasseri ,* and *Lactobacillus johnsonii*. Curr. Microbiol., 31: 114-118.

Prevost, H., and Divies, C., 1988. Continuous pre-fermentation of milk by entrapped yoghurt bacteria: development of the process. Milchwissenschaft 43: 621-625.

Psomas, E., Andrghetto, C., Litopoulou-Tzanetaki, E., Lombardi, A., Tzanetakis, N., 2001. Some probiotic properties of yeast isolates from infant feces and feta cheese. Int. J. Food Microbiol., 69: 125-133.

Rafsky, H. A., and Rafsky, J. C., 1955. Clinical and bacteriological studies of a new *Lactobacillus* concentrate in functional disturbances. Am. J. Gastroenterol., 24: 87–92.

Rangel, A., Domont, G. D., Pedrosa, C., and Ferreira, S. T., 2003. Functional properties of purified vicilins from cowpea (*Vigna unguiculata*) and pea (*Pisum sativum*) and cowpea protein isolate. J. Agric. Food Chem., 51: 5792-5797.

Rao, A. V., Shiwnarain, N. and Maharaj, J., 1989. Survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juices. Can. Inst. Food. Sci. Technol. J., 22: 345-349.

Rastall, R. A., Gibson, G. R., 2002. Prebiotic oligosaccharides: evaluation of biological activities and potential future developments. Caister Academic Press, London, 107-148.

Ray, B., 2004. Fundamental food microbiology, third edition. Boca Raton: CRC press

Redondo-Lopez, V., Cook, R. L., and Sobel, J. D., 1990. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Rev. Infect. Dis. 12: 856–872.

Reid, G., 2000. *In vitro* testing of *Lactobacillus acidophilus* NCFMTM as a possible probiotic for the urogenital tract. Int. Dairy J. 10: 415-419.

Reid, G., Jass, J., Sebulsky, M. T., and McCormick, J. K., 2003. Potential uses of probiotics in clinical practice. Clin. Microbiol. Rev., 16: 658-672.

Reid, G., McGroarty, J. A., Angotti, R. and Cook, R. L., 1988. *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. Can. J. Microbiol., 34: 344–351.

Rickard, A. H., Gilbert, P., High, N. J., Kolenbrander, P. E., and Handley, P. S., 2003. Bacterial coaggregation: an integral process in the development of multispecies biofilms. Trends Microbiol., 11: 94–100.

Riley, M. A., and Wertz, J. E., 2002. Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie., 84: 357-364.

Robinson, R. K., 1987. Survival of *Lactobacillus acidophilus* in fermented products. S. Afr. J. Dairy Sci., 19: 25-27.

Rojas, M., and Conway, P. L., 1996. Colonization by lactobacilli of piglet small intestinal mucus. J. Appl. Bacteriol., 81: 474-480.

Rolfe, R. D., 2000. The role of probiotic cultures in the control of gastrointestinal health. J. Nutr., 130: 396S-402S.

Rosa, M. L., Bottaro, G., Gulino, N., Gambuzza, F., Forti, F. D., Ini, G., and Tornambe, E., 2003. Prevention of antibiotic-associated diarrhea with *Lactobacillus sporogenes* and fructo-oligosaccharides in children. A multicentric double-blind vs placebo study (Italian). Minerva Pediatr., 55: 447-452.

Rowland, I. R., 1989. Metabolic profiles of intestinal floras. *In* Hattori, T., Ishida, Y., Maruyama, Y., Morita, R. Y., and Uchida, A., (ed.), Recent advances in microbial ecology. Japan Scientific Societies Press, Tokyo, Japan, 510-514.

Rowland, J. R., Rumney, C. J., Coytts, J. T., Lievense, L. C., 1998. Effect of *Bifidobacterium longum* and insulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. *Carcinogenesis,* 19: 281-285.

Roy, D. and Ward, P., 1990. Evaluation of rapid methods for differentiation of *Bifidobacterium* species. J. Appl. Bacteriol., 69: 139-149.

Ruiz-Moyano, S., Martin, A., Benito, M. J., Nevado, F. P., and Cordoba, M. G., 2008. Screening of lactic acid bacteria and bifidobacteria for potential probiotic use in Iberian dry fermented sausages. Meat Sci., 80: 715-721.

Rybka, S., and Kailaspathy, K., 1995. The survival of culture bacteria in fresh and freeze-dried AB yogurt. Aust. J. Dairy Tech., 50: 51-57.

Saarela, M., Lathenmaki, L., Crittenden, R., Salminen, S., and Mattilasandholm, T., 2002. Gut bacteria and health foods - the European perspective. Int. J. Food Microbiol., 78: 99-117.

Salminen, S., and Wright, V. A., 1998a. Lactic acid bacteria: microbiology and functional aspects, second edition, revised and expanded. New York: Marcel Dekker, Inc.

Salminen, S., Wright, V. A., Morelli, L., Marteau, P., Brassart, D., Vos de, W. M., Fonde'n, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S. E., and Sandholm, T. M., 1998b. Demonstration of safety of probiotics-a review. Int. J. Food Microbiol., 44: 93-106.

Savage, D. C., 1984. Adherence of the normal flora. In attachment of organisms to the gut mucosa. Vol. I. Beedeker, E. C. ed., Boca Raton CRC Press. p. 3-10.

Savage, D. C., 1992. Gastrointestinal microbial ecology; possible modes of action of direct-fed microbials in animal productiona review of the literature. In *Direct-fd Microbials in Animal Production* ed. National Feed Ingredients Association. West Des Meines, USA, 11-81.

Schellenberg, J., Smoragiewicz, W., and Karska-Wysocki, B., 2006. A rapid method combining immunofluorescence and flow cytometry for improved understanding of competitive interactions between lactic acid bacteria (LAB) and methicillin-resistant *S. aureus* (MRSA) in mixed culture. J. Microbiol. Meth., 65: 1-9.

Scheppach, W., Leuhrs, H., Menzel, T., 2001. Beneficial health effects of low-digestible carbohydrate consumption. Br. J. Nutr., 85: 823-930.

Schillinger, U., and Lucke, F. K., 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol., 55: 1901-1906.

Scott, K. P., 2002. The role of conjugative transposons in spreading antibiotic resistance between bacteria that inhabit the gastrointestinal tract. Cell. Mol. Life Sci., 59: 2071-2082.

Servin, A. L., 2004. Antagonistic activities of *Lactobacilli* and *Bifidobacteria* against microbial pathogens. FEMS Microbiol. Reviews, 28: 405-440.

Shah, N. P., and Rarula, R. R., 2000. Microencapsulation of probiotic bacteria and their survival in frozen fermented dairy desserts. Aust. J. Dairy Technol., 55: 139-144.

Shahani, K. M., Vakil, J. R., and Kilara, A., 1976. Natural antibiotic activity of *Lactobacillus acidophilus* and *bulgaricus*. II. Isolation of acidophilin from *L. acidophilus*. Cult. Dairy Prod. J., 12: 8-11.

Sheu, T. Y., and Marshall, R. T., 1991. Improving culture viability in frozen dairy desserts by microencapsulation. J. Dairy Sci., 74: 107.

Sheu, T. Y., and Marshall, R.T., 1993. Microentrapment of lactobacilli in calcium alginate gels. J. Food Sci., 54: 557-561.

Shin, H. S., Lee, J. H., Pestka, J. J., and Ustunol, Z., 2000. Growth and viability of commercial *Bifidobacterium* spp. in skim milk containing oligosaccharides and inulin. J. Food Sci., 65: 884-887.

Siigur, U., Tamm, E., Torm, S., Lutsar, I., Salminen, S., and Midtvedt, T., 1996. Effect of bacterial infection and administration of a probiotic on faecal short-chain fatty acids. Microbial Ecol. Health Dis., 9: 271-277.

Silva, M., Jacobus, N. V., Deneke, C. and Gorbach, S. L., 1987. Antimicrobial substance from a human *Lactobacillus* strain. Antimicrob. Agents Chemother., 31: 1231-1233.

Simon, G. L., and Gorbach, S. L., 1987. Intestinal flora and gastrointestinal function. In Physiology of Gastrointestinal Tract. 2nd ed. L. R. Johnson, ed. Raven Press, New York, NY. 2: 1729.

Simova, E. D., Beshkova, D. B., and Dimitrov, Z. P., 2009. Characterization and antimicrobial spectrum of bacteriocins produced by lactic acid bacteria isolated from traditional Bulgarian dairy products. J. Appl. Microbiol., 106: 692-701.

Smet, D., Hoorde, L.V., Woestyne, M. V., Christiaens, H., and Verstraete, V., 1995. Significance of bile salt hydrolytic activities of lactobacilli. J. Appl. Bacteriol., 79: 292-301.

Smith, T., 1995. The digestive system. The Human Body. Ken Fin Books, Collingwood, 150-173.

Solange, I., Ismael, M., and Mancilha, M., 2007. Non-digestible oligosaccharides: A review. Carbohydr. Polym., 68: 587-597.

Soomro, A. H, Masud T., and Anwaar, K., 2002. Role of lactic acid bacteria (LAB) in food reservation and Human Health- A Review. Pakistan J. Nutr., 1: 20-24.

Steenson, L. R., Klaenhammer, T. R., and Swaisgood, H. E., 1987. Calcium alginate-immobilized cultures of lactic streptococci are protected from attack by lytic bacteriophage. J. Dairy Sci., 70: 1121-1127.

Stewart, L., Pellegrini, C. A., Way, L. W., 1986. Antibacterial activity of bile acids against common biliary tract organisms. Surg. Forum, 37: 157-159.

Sultana, K., Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P., and Kailasapathy, K., 2000. Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. Int. J. Food Microbiol., 62: 47-55.

Suskovic, J., Kos, B., Goreta, J. and Matosic, S. 2001. Role of lactic acid bacteria and bifidobacteria in synbiotic effect. Food Technol. Biotechnol., 39: 227-235.

Swift, S., Karlyshev, A.V., Fish, L., Durant, E. L., Winson, M.K., Chhabra, S. R., Williams, P., Macintyre, S., and Stewart, G. S. A. B., 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the *Lux*RI homologs *Ahy*RI and *Asa*RI and their cognate N-acylhomoserine lactone signal molecules. J. Bacteriol. 179: 5271-5281.

Szilagyi, A., 2004. Redefining lactose as a conditional prebiotic. Can. J. Gastroenterol., 218: 163-167.

Tannock, G. W., Dashkevicz, M. P. and Feighner, S. D., 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. Appl. Environ. Microbiol., 55: 1848-1851.

Tannock, G. W., Munro, K., Harmsen, H. J. M., Welling, G. W., Smart, J., and Gopal, P. K., 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Appl. Environ. Microbiol., 66: 2578-2588.

Tappenden, K. A. and A. S. Deutsch, 2007. The physiological relevance of the intestinal microbiota - contributions to human health. J. Am. Coll. Nutr., 26: 679S-683S.

Taylor, A. L., Dunstan, J. A., and Prescott, S. L., 2007. Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. J. Allergy Clin. Immunol., 119: 184-191.

Teitelbaum, J. E., and Walker, W. A., 2002. Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. Ann. Rev. Nutr., 22: 107-138.

Torriani, S., Felis, G. E., and Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum, L. pentosus,* and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. Appl. Environ. Microbiol., 67: 3450-3454.

Toure, R., Kheadr, E., Lacroix, C., Moroni, O., and Fliss, I. 2003. Production of antibacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogenes.* J. Appl. Microbiol., 95: 1058-1069.

Tuomola, E. M., and Salminen, S. J., 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int. J. Food Microbiol., 41: 45-51.

Vandenbergh, P., 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiol. Rev., 12: 221-238.

Velraeds, M. M. C., Mei, H. C. V. D., Reid, G., and Busscher, H. J., 1996. Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. Appl. Environ. Microbiol., 62: 1958–1963.

Ventura, M., Casas, I. A., Morelli, L., and Callegari, M. L., 2000. Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of *Lactobacillus* spp. isolated from fecal and vaginal samples. Syst. Appl. Microbiol., 23: 504-509.

Vernazza, C. L., Gibson, G. R., and Rastall, R. A., 2006. Carbohydrate preference, acid tolerance, and bile tolerance in five strains of *Bifidobacterium*. J. Appl. Microbiol., 100: 846-853.

Vincent, J. G., Veomett, R. C., and Riley, R. F., 1959. Antibacterial activity associated with *Lactobacillus acidophilus.* J. Bacteriol., 78: 477– 484.

Vinderola, C. G., Medici, M., and Perdig, G., 2004. Relationship between interaction sites in the gut, hydrophobicity, mucosal immunomodulating capacities and cell wall protein profiles in indigenous and exogenous bacteria. J. Appl. Microbiol., 93: 794–799.

Vine, N. G., Leukes, W. D., Kaiser, H., Daya, S., Baxter, J., and Hecht, T., 2004. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. J. Fish Dis., 27: 319-326.

Walter, J., Hertel, C. Tannock, G. W., Lis, C. M., Munro, K., and Hammes, W. P., 2001. Detection of *Lactobacillus, Pediococcus, Leuconostoc,* and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol., 67: 2578-2585.

Wang, Y., 2009. Prebiotics: Present and future in food science and technology. Food Res. Int., 42: 8-12.

Ward, D. M., Weller, R., and Bateson, M. M., 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature, 345: 63-65.

Wenrong, S. and Griffiths, M. W., 2000. Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan-xanthan beads. Int. J. Food Microbiol., 61: 17-26.

Westerlund, B. and Korhonen, T. K., 1993. Bacterial proteins binding to the mammalian extracellular matrix. Mol. Microbiol., 9: 687-694.

Zavaglia, A. G., Kociubinski, G., Perez, P., Disalvo, E., and Antoni, D. G., 2002. Effect of bile on the lipid composition and surface properties of bifidobacteria. J. Appl. Microbiol., 93: 794-799.

Zboril, V., 2002. Physiology of microflora in the digestive tract. Vnitr. Lek., 48: 17–21.

Zhou, J. S., Pillidgec, C. J., Gopal P. K., and Gill, H. S., 2005. Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. Int. J. Food Microbiol., 98: 211-217.

Ziemer, C. J., and Gibson, G. R., 1998. An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. Int. Dairy J., 8: 473-479.

APPENDIX A

A.1 Screening for probiotic co-aggregation with other intestinal flora

A.1.1 Bacterial adhesion to hydrocarbons

The percent adhesions of common enteric pathogens like *Salmonella enteridis* and *Escherichia coli* O157:H7, commensal microflora of GIT including *Escherichia coli* ATCC 14763, *Escherichia coli* ATCC 4351, *Enterococcus fecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, and tested probiotics are shown in Figure A.1.

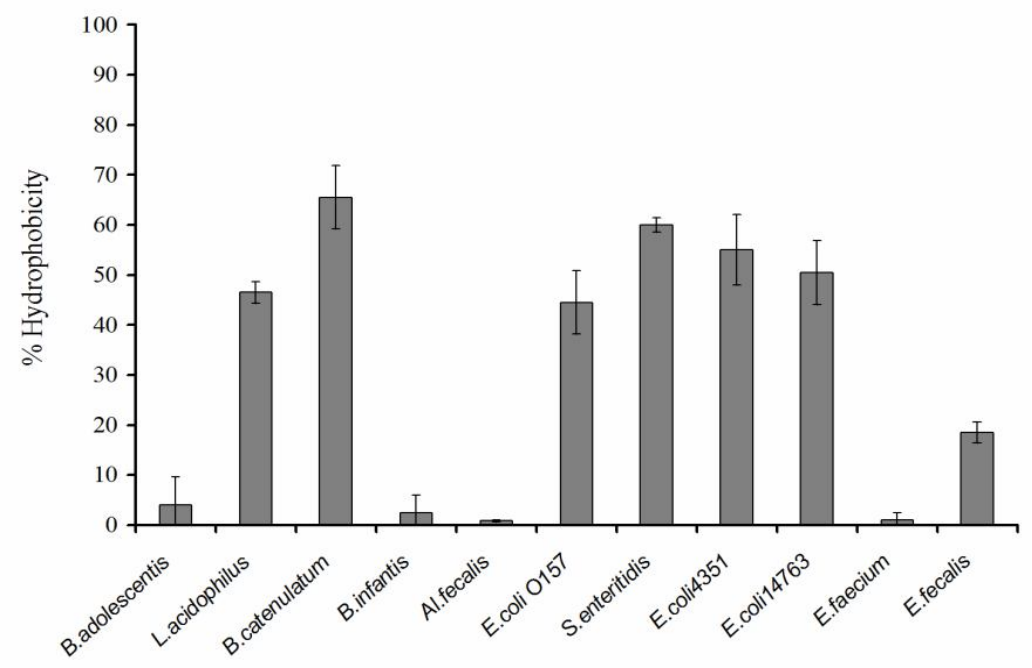

Figure A.1 Hydrophobicity of probiotic and pathogen strains expressed as percentages. The data are expressed as the mean ± one standard deviation (n=3)

The effect of $MgCl_2$, $CaCl_2$, EDTA and oxgall bile on cell surface hydrophobicity of probiotics is shown in Figure A.2. Cell surface hydrophobicity of all tested probiotics was found to be reduced as the concentration of $MgCl_2$ was increased from 0.5-5 mM, and EDTA concentration from 100-500 µM. Addition of $CaCl_2$ (0.5-10 mM) in MRS media was found to

increase the cell surface hydrophobicity of all tested probiotics. Interestingly, the percent hydrophobicity decreased with the addition of Oxgall bile. Increase in bile concentration from 0.1-0.5% (w/v) caused a further decrease in hydrophobicity.

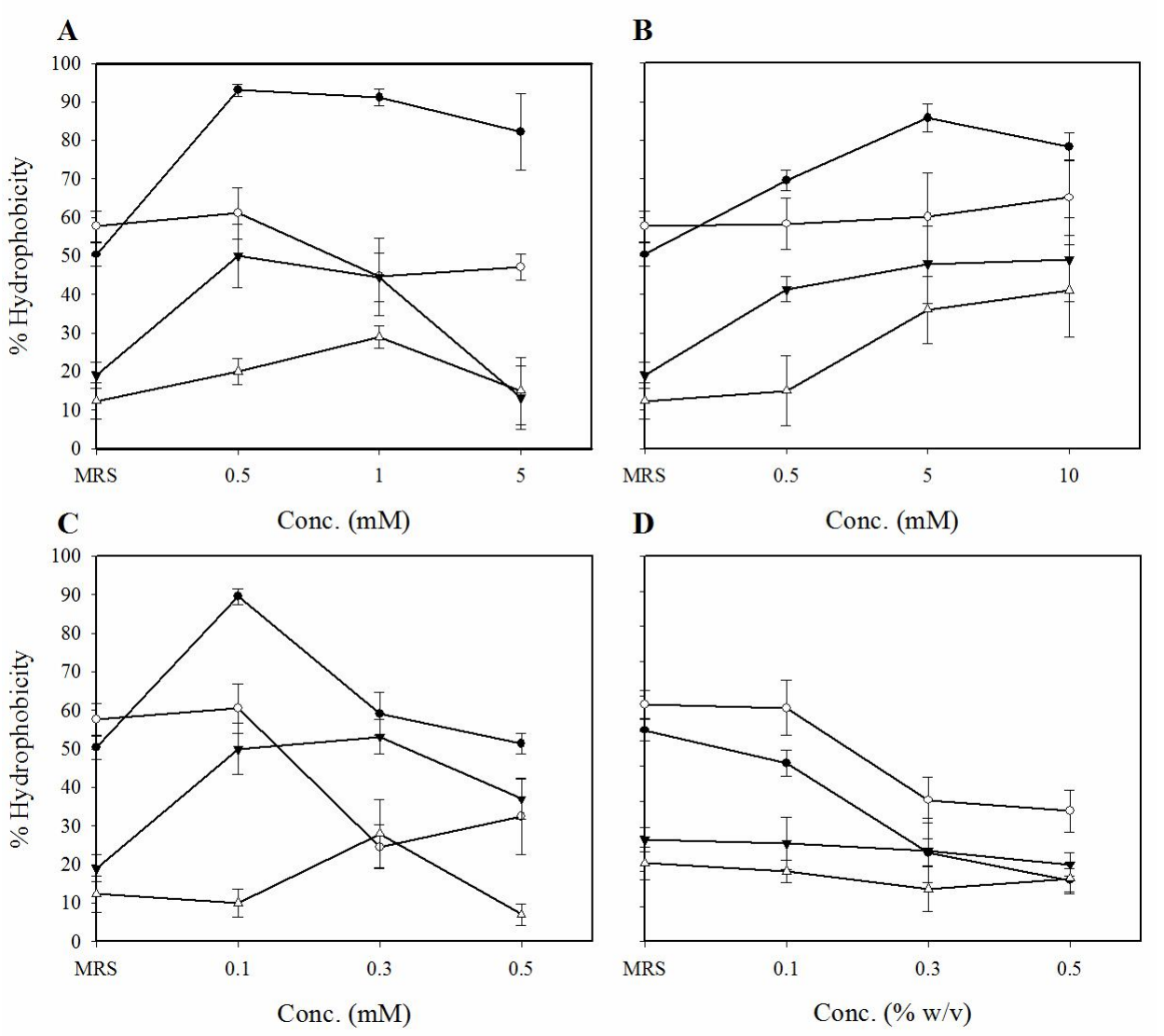

Figure A.2 Effect of different concentrations of A) $MgCl_2$, B) $CaCl_2$, C) EDTA and D) 0.3% Oxgall bile (w/v) on cell surface hydrophobicity of *L. acidophilus* (●), *B. catenulatum* (○), *B. adolescentis* (▼) and *B. infantis* (Δ). The data are expressed as the mean ± one standard deviation (n=3)

A.1.2 Bacterial auto-aggregation analysis

The auto-aggregation properties of probiotic and pathogen strains were tested over a period of 24 h by visual observation of aggregates formed as well as by absorbance measurements. The results obtained by absorbance measurements are shown in Figure A.3.

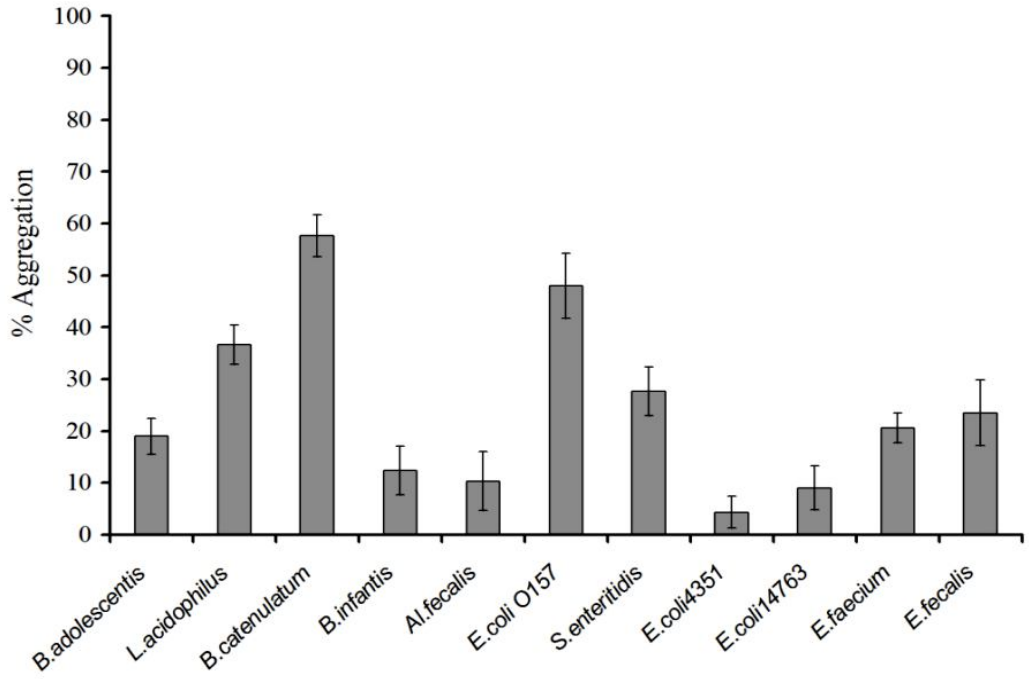

Figure A.3 Autoaggregation of probiotic and pathogen strains expressed as percentages. The data are expressed as the mean ± one standard deviation (n=3)

In general, the probiotic strains presented higher auto-aggregation abilities than the pathogens when examined at 37°C. This temperature was chosen because 37°C is the normal body temperature. The pathogen with the greatest auto-aggregation potential was *E. coli* O157, whereas the probiotic strain with the greatest auto-aggregation potential was *B. catenulatum.* BATH values (Figure A.1) were related to auto-aggregation properties (Figure A.3) because most of the strains with higher adhesion to hydrocarbons also showed high auto-aggregation abilities. Among the tested probiotic strains, *L. acidophilus* and *B. catenulatum* showed highest

percent adherence to hydrocarbon (% BATH) and also highest auto-aggregation abilities. *B. adolescentis* and *B. infantis* showed a lower % BATH as well as lower autoaggregation.

A.1.3 Visual co-aggregation analysis

Visual co-aggregation of two probiotic strains in combination with other intestinal flora, including *E. coli* 4351, *E. coli* 14763, *Alcaligens fecalis*, *E. faecium* and *E. fecalis* and pathogen strains is shown in Table A.1. All tested probiotics showed co-aggregation with some of the commensal and pathogenic bacteria in the test panel. It was interesting to note that incubation of two probiotic strains with a single pathogen strain resulted in greater co-aggregation and more rapid and complete clearing of liquid in the culture tube. Figure A.4 shows photomicrographs of the precipitated floccules.

Table A.1 Coaggregation of different combinations of probiotic bacteria with enteric pathogens and commensal bacteria

	Probiotic combinations					
Bacteria	LA+BA	LA+BC	LA+BI	BA+BC	BA+BI	BC+BI
E. coli 4351	+++	++++	+++	+	++++	++++
E. coli 14763	+++	+++	++	++	++++	++++
E. coli O157	++	+++	++	++++	++	++++
S. enteritidis	++++	++++	++	++++	++	++++
Al. fecalis	++++	++++	+++	++++	++++	++++
E. faecium	++++	++++	+++	++++	++++	++++
E. fecalis	+++	++++	+++	++++	++++	++++

LA, *L. acidophilus*; BA, *B. adolescentis*; BC, *B. catenulatum*; BI, *B. infantis*
(+) for small uniform aggregates in the suspension, (++) for aggregates that are easily seen but do not settle, (+++) for large aggregates which settle and leave some turbidity in the supernatant fluid, and (++++) for large aggregates which settle and leave clear supernatant fluid.

The formation of aggregates was dependant on the strains tested (probiotic and pathogen strains) and also the concentration and time of co-incubation. Formation of aggregates was found

to be dependant on the concentration of pathogen and probiotic strains. It was noted that when the concentration of pathogen was lower than the probiotic, complete clearing of liquid in the culture tube containing combination of probiotics and pathogen was observed. This finding supports the idea that given the low infective dose of certain pathogens, co-aggregation with commensal or probiotic microbiota could potentially help wash them from the GIT, thus preventing their colonization and manifestation of illness.

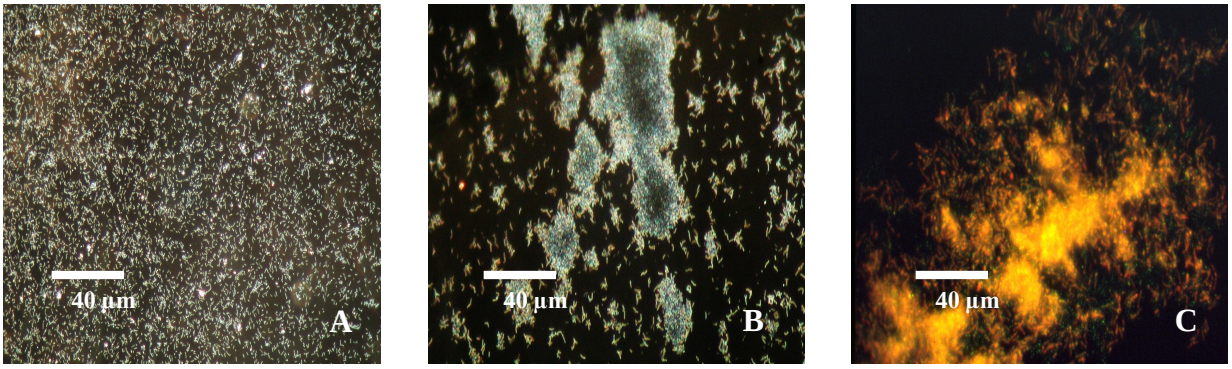

Figure A.4 Photomicrographic images of co-aggregates of *L. acidophilus*, *B. adolescentis* and *S. enteritidis* in combination; A) Unstained bacteria before incubation, B) Unstained co-aggregates after incubation at 37ºC for 24 h, and C) Fluorescently-stained (*Bac*Light™ Live/Dead stain) co-aggregates